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New molecular strategies for prevention of fibroproliferative vascular disease

- An experimental approach to restenosis

Nina-Maria Tigerstedt, MD

Transplantation Laboratory, University of Helsinki and
Helsinki University Central Hospital,
Helsinki, Finland.

ACADEMIC DISSERTATION

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Supervised by

Hanna Savolainen-Peltonen, MD, PhD
Transplantation Laboratory, University of Helsinki and
Helsinki University Central Hospital
Helsinki, Finland

and

Professor Pekka Häyry, MD, PhD, FACS (Hon.)
Transplantation Laboratory, University of Helsinki and
Helsinki University Central Hospital
Helsinki, Finland, and
Department of Surgery and Pathology,
University of Calgary
Calgary, AL, Canada

Reviewed by

Professor Timo Paavonen, MD, PhD
Department of Pathology
University of Tampere
Tampere, Finland

and

Docent Maarit Venermo, MD, PhD
Department of Vascular Surgery
Helsinki University Central Hospital
Helsinki, Finland

Discussed with

Professor Markku S. Nieminen, MD, PhD
Division of Cardiology
Helsinki University Central Hospital
Helsinki, Finland

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ORIGINAL PUBLICATIONS

This Thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Tigerstedt NM, Savolainen-Peltonen H, Lehti S, Häyry P. Vascular cell kinetics in response to intimal injury *ex vivo*. Journal of Vascular Research. 2009; in press.

II Aavik E, Luoto NM, Petrov L, Aavik S, Patel Y, Häyry P. Elimination of vascular fibrointimal hyperplasia by somatostatin receptor 1,4 selective agonist in rat. FASEB Journal. 2002; 16: 724-726. *

III Tigerstedt NM, Aavik E, Aavik S, Savolainen-Peltonen H, Häyry P. Vasculoprotective effects of somatostatin receptor subtypes. Molecular and Cellular Endocrinology. 2007; 279: 34-38.

IV Tigerstedt NM, Aavik E, Lehti S, Häyry P, Savolainen-Peltonen H. Synergistic effect of sirolimus and imatinib in preventing restenosis after intimal injury. Journal of Vascular Research. 2009; 46: 240-252.

* This publication has also been used in the Thesis of Einari Aavik (2005) with the permission of the Medical Faculty of the University of Helsinki.

ABBREVIATIONS

ACE	angiotensin-converting enzyme
bFGF	basic fibroblast growth factor
BMS	bare metal stent
BrdU	5-bromo-2'-deoxyuridine
CPM	counts per minute
CSRP2	Cystein and glycine-rich protein 2
CYP	Cytochrome P450
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
FKBP	FK506 binding protein
GPCR	G-protein coupled receptor
^{3}H-TdR	tritiated thymidine
hCAEC	human coronary artery endothelial cells
HEV	high endothelial venule
HUVEC	human umbilical vein endothelial cells
IGF-1	insulin-like growth factor-1
IL	interleukin
LCA	leukocyte common antigen
MAPK	mitogen-activated protein kinase
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
mTOR	mammalian target of rapamycin
PDGF	platelet derived growth factor
PDGF-R	platelet derived growth factor receptor
PTCA	percutaneous transluminal coronary angioplasty
PTK	protein tyrosine kinase
QRT-PCR	quantitative real-time polymerase chain reaction
RIA	radioimmunoassay
SCF	stem cell factor
SES	sirolimus-eluting stent
SMA	smooth muscle α-actin
SMC	smooth muscle cell
SST-14	somatostatin-14
SST-18	somatostatin-18
sst_1-sst_5	somatostatin receptor subtypes 1 through 5
TGF-ß	transforming growth factor-ß
vWF	von Willebrandt factor

ABSTRACT

Vascular intimal hyperplasia is a major complication following angioplasty. The hallmark feature of this disorder is accumulation of dedifferentiated smooth muscle cells (SMCs) to the luminal side of the injured artery, cellular proliferation, migration, and synthesis of extracellular matrix. This finally results in intimal hyperplasia, which is currently considered an untreatable condition. According to current knowledge, a major part of neointimal cells derive from circulating precursor cells. This has outdated the traditional *in vitro* cell culture methods of studying neointimal cell migration and proliferation using cultured medial SMCs.

Somatostatin and some of its analogs with different selectivity for the five somatostatin receptors (sst_1 through sst_5) have been shown to have vasculoprotective properties in animal studies. However, clinical trials using analogs selective for sst_2/sst_3/sst_5 to prevent restenosis after percutaneous transluminal coronary angioplasty (PTCA) have failed to show any major benefits. Sirolimus is a cell cycle inhibitor that has been suggested to act synergistically with the protein-tyrosine kinase inhibitor imatinib to inhibit neointimal hyperplasia in rat already at well-tolerated submaximal oral doses. The mechanisms behind this synergy and its long-term efficacy are not known.

The aim of this study was to set up an *ex vivo* vascular explant culture model to measure neointimal cell activity without excluding the participation of circulating progenitor cells. Furthermore, two novel potential vasculoprotective treatment strategies were evaluated in detail in rat models of neointimal hyperplasia and in the *ex vivo* explant model: sst_1/sst_4-selective somatostatin receptor analogs and combination treatment with sirolimus and imatinib.

This study shows how whole vessel explants can be used to study the kinetics of neointimal cells and their progenitors, and to evaluate the anti-migratory and anti-proliferative properties of potential vasculoprotective compounds. It also shows how the influx of neointimal progenitor cells occurs already during the first days after vascular injury, how the contribution of cell migration is more important in the injury response than cell proliferation, and how the adventitia actively contribute in vascular repair.

The vasculoprotective effect of somatostatin is mediated preferentially through sst_4, and through inhibition of cell migration rather than of proliferation, which may explain why sst_2/sst_3/sst_5-selective analogs have failed in clinical trials. Furthermore, a brief early oral treatment with the combination of sirolimus and imatinib at submaximal doses results in long-term synergistic suppression of neointimal hyperplasia. The synergy is a result of inhibition of post-operative thrombocytosis and leukocytosis, inhibition of neointimal cell migration to the injury-site, and maintenance of cell integrity by inhibition of apoptosis and SMC dedifferentiation.

In conclusion, the influx of progenitor cells already during the first days after injury and the high neointimal cell migratory activity underlines the importance of early therapeutic intervention with anti-migratory compounds to prevent neointimal hyperplasia. Sst_4-selective analogs and the combination therapy with sirolimus and imatinib represent potential targets for the development of such vasculoprotective therapies.

INTRODUCTION

Atherosclerosis and vasculoproliferative disorders are major health concerns, causing excess humane suffering, having great economical consequences, and contributing to over half of the deaths in the Western World (Braunwald 1997). PTCA is used widely in coronary heart disease patients to restore the circulation in atherosclerotic coronary arteries; it is, however, complicated by restenosis in 30% to 50% of the patients (Holmes et al. 1984). Allograft arteriosclerosis is a major manifestation of chronic rejection, limiting the long-term success rate in transplantation. Furthermore, accelerated generalized arteriosclerosis contributes to death with a functioning graft (Ojo et al. 2000, Kasiske 2001).

Characteristic to vasculoproliferative disorders is the accumulation of SMCs to the inner side of the injured artery, their proliferation, migration, and synthesis of extracellular matrix. This results in neointima formation, vascular remodeling, and finally, vascular stenosis and ischemia of the tissues perfused by these vessels (Ross 1995). Still, the pathogenesis of these vasculoproliferative processes is largely unknown and despite extensive efforts to target medial SMCs, the treatment options are limited. However, according to current knowledge, a remarkable part of the neointimal cells derive from circulating precursor cells (Saiura et al. 2001). This outdates the common *in vitro* methods of studying neointimal cells using cultured medial SMCs, and creates a need for new approaches in the research of vasculoproliferative disorders.

Somatostatin is a vasculoprotective neurohormone that exerts its action through five G-protein-coupled receptors (GPCRs), sst_1 through sst_5 (Lundergan et al. 1991, Reisine and Bell 1995). Despite promising results from animal experiments (Lundergan et al. 1991, Howell et al. 1993), clinical trials evaluating the effects of sst_2, sst_3, and sst_5-selective agonists octreotide and lanreotide in inhibiting restenosis were not successful (Eriksen et al. 1995, von Essen et al. 1997). However, the predominant receptor subtypes in rat carotid arteries are sst_1 and sst_4, and after denudation injury their expression increases acutely, whereas the expression of sst_2 and sst_5 remains low (Khare et al. 1999). Also, in human atherosclerotic vessels the predominant receptor subtypes are sst_1 and sst_4 (Curtis et al. 2000). Thus, wrong receptor subtypes might have been targeted in the clinical trials.

Sirolimus (rapamycin, Rapamune®) is a powerful antiproliferative agent that inhibits cell cycle progression mainly through inhibition of mammalian target of rapamycin (mTOR) (Abraham et al. 1996). Sirolimus is currently used in drug-eluting stents for the prevention of restenosis after PTCA (Morice et al. 2002). However, the safe use of sirolimus-eluting stents (SESs) is limited to very specific vessel and lesion types (Laskey et al. 2007). Oral use of sirolimus has been shown to inhibit intimal hyperplasia after denudation injury in animal models (Gregory et al. 1993a), but its clinical use has been limited by the side-effects associated with the high doses needed for inhibition of restenosis (Rodriguez et al. 2003).

Imatinib mesylate (STI-571, CGP 57148B, Glivec®, Gleevec®) is a protein-tyrosine kinase inhibitor in clinical use for cancer treatment. In experimental studies imatinib has shown vasculoprotective properties (Myllärniemi et al. 1999, Sihvola et al. 1999).

Recently, the combination of imatinib and sirolimus was shown to work synergistically in preventing restenosis after balloon-injury in rat already at sub-maximal oral doses (Vamvakopoulos et al. 2006). The mechanisms behind this synergy and its long-term efficacy are not known.

The aim of this study was to develop an *ex vivo* explant culture model to study neointimal cell movement, proliferation and differentiation also taking into consideration the participation of circulating progenitor cells. Moreover, this model was used together with an *in vivo* experimental restenosis model to evaluate potential new vasculoprotective compounds, including sst_1- and sst_4-selective agonists and the sirolimus-imatinib combination.

REVIEW OF THE LITERATURE

1. Vasculoproliferative disorders

1.1 Atherosclerosis

Atherosclerosis is a systemic inflammatory disease of the vascular wall (Schwartz et al. 1985, Ross 1993, 1999). Essential in the development of atherosclerosis is endothelial injury, which may be caused by hyperlipidemia, hypertension, or by agents such as the herpes simplex virus (Yamashiroya et al. 1988), Chlamydia pneumoniae (Saikku et al. 1988, Carlsson et al. 1997), or tobacco smoke toxins (Shinton et al. 1989).

Atherosclerotic leasions consist mostly of inflammatory cells, monocyte-derived macrophages, and T lymphocytes. Later on, lipids start to accumulate within the macrophages and SMCs are also present in the lesions (Stary et al. 1994). If the atherosclerotic changes progress, these initial fatty streaks, which can already be observed in early childhood (Napoli et al. 1997), develop into more advanced lesions: plaques with a core of lipids and necrotic tissue, covered by a fibrous cap (Stary et al. 1995). Plaque rupture or endothelial erosion can initiate thrombosis and arterial occlusion, causing an acute coronary syndrome, a stroke, or ischemia of the lower limb, depending on the location of the affected artery.

Well known risk factors for atherosclerosis are e.g. smoking, hyperlipidemia, physical inactivity, hypertension, diabetes, aging, male gender and familial predisposition. Primary prevention of atherosclerosis includes maintaining a healthy life-style and risk-intervention, such as blood pressure control, blood lipid management, avoidance of tobacco smoke, weight management, diabetes management, and low-dose aspirin for high-risk patients (Pearson et al. 2002). Secondary prevention in patients with cardiovascular disease may also include treatment with clopidogrel or warfarin, angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers, and beta blockers (Smith et al. 2006).

In severe cases, surgical revascularisation is indicated to relieve symptoms of ischemia and to maintain tissue vitality. The most common treatment choices are angioplasty with or without stenting, or bypass surgery. Each of these procedures carry their risks and limitations, and none of them can be considered as a permanent cure for atherosclerosis.

1.2 Restenosis

PTCA, a procedure where a stenosed or occluded coronary is expanded with an inflatable balloon catheter, was first introduced in 1977 (Gruntzig et al. 1977). Similar techniques are also used to treat carotid artery stenosis and occlusions in arteries of the lower extremities. Restenosis, the re-narrowing of an occluded or stenosed artery treated by

angioplasty, is a major limitation for the long-term success of this procedure, and as many as 30-50% of vessels are re-occluded six months after PTCA (Holmes et al. 1984, Gruentzig et al. 1987, Nobuyoshi et al. 1988).

In the development of restenosis, a critical event is the mechanical injury to the luminal endothelial layer caused by the angioplasty balloon catheter. This triggers a repair response characterized by neointimal hyperplasia - the migration and proliferation of SMCs and extracellular matrix deposition - and vessel remodeling. This leads to the re-occlusion, or restenosis, of the vessel, worsening the clinical situation and creating a need for either a new procedure or bypass operation.

The risk factors for restenosis are poorly identified and hard to predict. Patient-related factors such as diabetes, hypertension, hypercholesterolemia, renal disease, as well as certain lesion and vessel types, have been associated with a higher restenosis risk (Kahn et al. 1990, Reis et al. 1991, Hermans et al. 1993, Weintraub et al. 1993).

A great deal of research has focused on the prevention of restenosis after PTCA (Table 1). Trials with systemic glucocorticoid therapy, angiotensin converting enzyme inhibitors, heparin, and statins have not been successful (Pepine et al. 1990, Faxon 1995, Karsch et al. 1996, Serruys et al. 1999). Experiences with devices releasing gamma or beta radiation (brachytherapy) at the denudation site have also been discouraging in long-term follow ups (Ferrero et al. 2007). Somatostatin-based therapies have exhibited variable success, and these therapies are discussed in detail on page 24.

Table 1 *Some experiences from randomized clinical trials on the treatment of restenosis.*

Target/compound	**Study size (patients)**	**Restenosis rate at 6 months**	**Reference**
Coagulation pathways			
LMW heparin	625	33% vs 34.4%, p=NS	Karsch et al. 1996
ACE			
Cilazapril	1436	34-40% vs 33%*, p=NS	Faxon 1995
HMG-CoA reductase			
Fluvastatin	1054	28% vs 31%, p=NS	Serruys et al. 1999
Simvastatin + BMS	525	25.4% vs 38%, p<0.005	Walter et al. 2000
Glucocorticoid receptors			
Methylprednisolone	915	40% vs 39%, p=NS	Pepine et al. 1990
sst_2/sst_3/sst_5			
Lanreotide	553	12% vs 40%, p=0.003	Emanuelsson et al. 1995
Lanreotide	112	36% vs 37%, p=NS	Eriksen et al. 1995
Octreotide	217	34.3% vs 33.9%, p=NS	von Essen et al. 1997
mTOR			
Sirolimus + BMS	100	12% vs 34.6%**, p=0.009	Rodriguez et al. 2006
SES	1058	3.2% vs 35.4%**, p<0.001	Moses et al. 2003
SES	238	0% vs 26.6%, p<0.001	Morice et al. 2002

* results depended on the drug dosage; ** follow-up at 9 months

A significant improvement in the treatment of restenosis was clearly the introduction of bare metal stents (BMSs) in 1994. Inserted into the artery during the PTCA to prevent negative remodeling and elastic recoil, BMSs help to maintain blood flow through the artery (Fischman et al. 1994, Serruys et al. 1994). The success of stenting is, however, limited by in-stent restenosis (Fischman et al. 1994, Serruys et al. 1994). Lately, the use of stents coated with antiproliferative drugs, such as sirolimus and paclitaxel, have been predicted to revolutionize the prevention of restenosis after stenting (Liistro et al. 2002, Morice et al. 2002, Grube et al. 2003). However, concern has been raised about the safety, long-term efficacy and cost-effectiveness associated with these stent types (Daemen et al. 2007, Kastrati et al. 2007).

1.3 Transplant arteriosclerosis

Transplant arteriosclerosis is the main manifestation of chronic allograft rejection. While modern immunosuppressive protocols have raised the one-year graft survival rate to over 90% for heart and kidney transplants (Orens et al. 2006, Cecka 1997), transplant arteriosclerosis remains the major cause for poor long-term success of transplanted organs (Sharples et al. 1991, Paul 1993). The incidence of transplant arteriosclerosis is highest in cardiac grafts, where changes due to transplant arteriosclerosis can be seen in up to 60% of the grafts at one year after transplantation (Julius et al. 2000). Furthermore, in kidney transplant recipients accelerated generalized atherosclerosis accounts for a significant number of deaths with a functioning graft (Ojo et al. 2000, Kasiske 2001).

In the development of transplant arteriosclerosis endothelial dysfunction is caused to a great extent by a chronic inflammatory injury. The infiltration of inflammatory cells into graft arteries results in formation of neointimal fibrosis and vascular remodeling, which finally leads to constriction of the vascular lumen and graft ischemia (Uys et al 1984, Billingham 1987, Shi et al. 1996a). The changes in the arterial wall resemble those of classical atherosclerosis, but they affect the entire length of the vessel, and the lesions are diffuse and concentric (Billingham 1989, Shi et al. 1996a), rather than focal and eccentric as in atherosclerosis (Fig. 1). Also, lipid accumulations are less common in the early stages of transplant arteriosclerosis (Pucci et al. 1990), and the disease progresses faster (Paul 1993).

The risk factors for chronic allograft rejection are still incompletely known, but include a high donor age, acute rejection, and a long cold ischemia time (Rao et al. 1990, Basadonna et al. 1993, Schwartz et al. 2005). Different immunosuppressive regimens improve long-term graft survival, but there is no known effective treatment available for transplant arteriosclerosis.

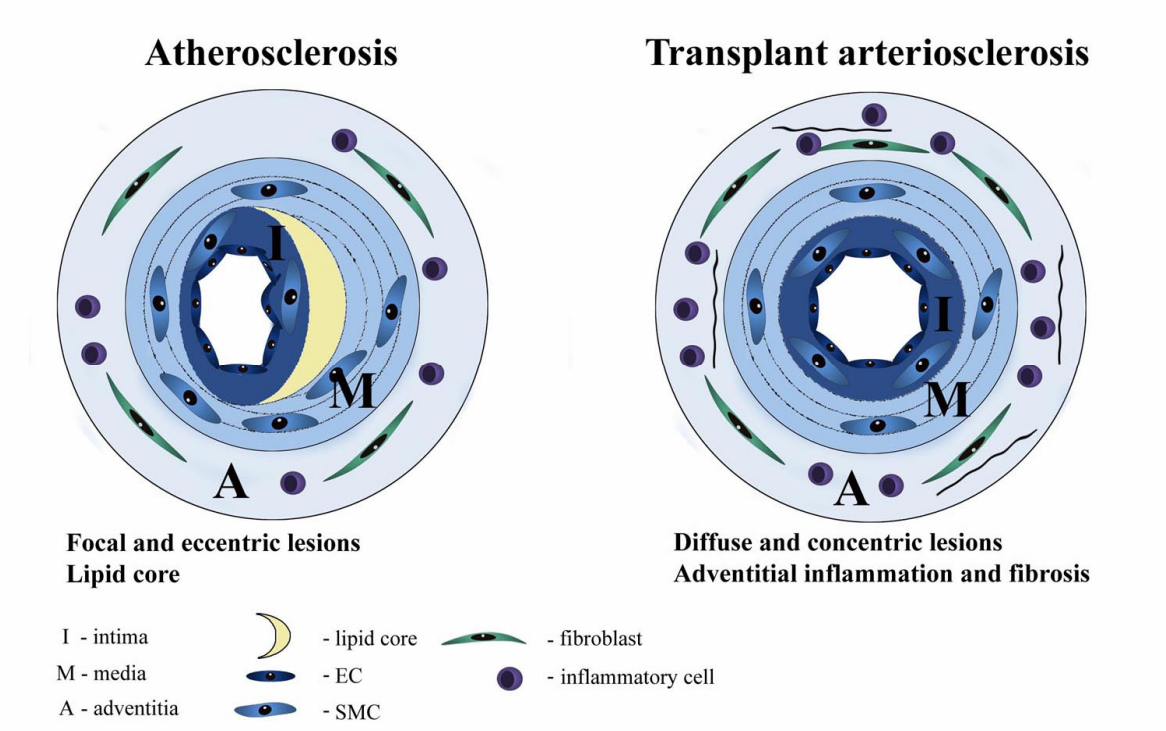

Figure 1 *A schematic presentation of the differences between atherosclerosis and transplant arteriosclerosis.*

2. Pathogenesis of vascular stenosis

2.1 Structure and function of the arterial wall

The human arterial wall is composed of three layers, the intima, media, and adventitia. The innermost layer, the intima, consists of a single layer of endothelial cells on a basal lamina, connective tissue, and the internal elastic lamina. The intima serves as a barrier, controlling the movement of cells and other substances from the bloodstream into the arterial wall, and further, into other tissues. The endothelium also maintains the inner side of the artery in an anti-thrombotic state, a function that can easily be lost as a consequence of endothelial injury. Furthermore, endothelial cells serve as regulators of inflammatory reactions, and control the growth of other cells types, such as SMCs (Ross and Pawlina 2006). After vascular injury, re-endothelialization of the site of injury is a crucial process in arterial repair (Clowes et al. 1986).

The middle layer, the media, consists of layers of circumferentially arranged SMCs. The media preserves the elasticity of the arteries, enabling them to adjust to blood flow pressure. The major task of the differentiated medial SMCs is vasoconstriction and vasodilatation. Traditionally, SMCs have been thought to contribute also to arterial wall repair, and pathological SMC accumulation is a characteristic feature of intimal hyperplasia. SMC activity is regulated by several growth promoters, such as platelet-

derived growth factor (PDGF) produced by platelets, endothelial cells and macrophages, basic fibroblast growth factor (bFGF), and interleukin 1 (IL-1) (Schwartz et al. 1986, Ross and Pawlina 2006).

The outermost layer, the adventitia, consists of connective tissue, and is separated from the media by the external elastic lamina. The adventitia with its network of elastic fibers and collagen provides support for the arterial wall, helping the vessel to resist the pressure of the blood flow. It also contains the *vasa vasorum*, small blood vessels supplying the outer parts of the vessel wall. The inner parts of the vascular wall are supplied from the luminal side (Ross and Pawlina 2006). More recently, adventitial myofibroblasts have been suggested to contribute actively to neointimal development by proliferating and possibly by migrating to the neointima following vascular injury (Scott et al. 1996, Shi et al. 1996b, Zalewski and Shi 1997, Oparil et al. 1999, Couffinhal et al. 2001, Frosen et al. 2001). Adventitial leukocyte accumulation has implied a role for adventitial inflammation in vascular lesion formation (Hayashi et al. 2000). Furthermore, increased numbers of adventitial microvessels have been reported in several diseases affecting the vasculature, such as vasculitis and hypercholesterolemia (Folkman 1995, Kwon et al. 1998, Kaiser et al. 1999). In a porcine model, the number of adventitial microvessels has been shown to increase acutely after denudation injury (Pels et al. 1999) and stenting (Cheema et al. 2006), although the precise role of these vessels in vascular repair is still to be elucidated. The adventitia has also been suggested as a site of entrance, or reserve, for neointimal progenitor cells (Hu et al. 2004).

2.2 The response to vascular injury

Central in our understanding of all vasculoproliferative disorders is the response-to-injury hypothesis: injuries to the luminal endothelium trigger a cascade of events eventually leading to the development of intimal thickening and vascular remodeling (Libby et al. 1992, Häyry et al. 1993a, Ross 1995, 1999). This physiological healing process, initially designed to guarantee undisrupted blood flow through the artery, can become exaggerated, and paradoxically lead to arterial changes causing vessel occlusion and ischemia in the area that the vessel is supplying.

The common denominator for all vasculoproliferative diseases is an injury to the endothelium. This results in endothelial dysfunction, increases endothelial permeability, and provokes an inflammatory response (Ross 1999). The injury increases the adhesiveness of the endothelium for platelets and leukocytes, and induces an upregulation of a variety of adhesion molecules. These include selectins (Larsen et al. 1989, McEver et al. 1989), integrins (Diacovo et al. 1996, Koyama et al. 1996), platelet-endothelial-cell adhesion molecule-1 (Woodfin et al. 2007), intercellular adhesion molecule-1, and vascular-cell adhesion molecule-1 (Sluiter et al. 1993, Tanaka et al. 1993), mediating the rolling of leukocytes at the site of injury, as well as the adhesion of leukocytes to endothelial cells. Thereafter, leukocytes transmigrate into the vascular wall, a process mediated by several factors, including the monocyte chemoattractant protein-1 (Rollins

1997, Mukaida et al. 1998, Usui et al. 2002), osteopontin (Scatena et al. 2007), interleukins (Dinarello 1996), and PDGF (Deuel et al. 1982, Williams et al. 1983).

Leukocyte activation results in the secretion of metalloproteinases, growth factors, inflammatory mediators, and tissue factor. These agents cause inflammatory endothelial damage directly, trigger vasoconstriction, promote SMC proliferation, recruit mononuclear monocytes, stimulate platelet activation and aggregation, and directly initiate the extrinsic coagulation pathway (Bazzoni et al. 1991, Ricevuti et al. 1991).

Mechanical stretch, exposure to circulating mitogens, as well as growth factors and cytokines released from platelets, endothelial cells, and inflammatory cells result in SMC migration to the site of injury, and proliferation to form atherosclerotic lesions, restenosis or transplant arteriopathy (Libby et al. 1992, Häyry et al. 1993a, Ross 1999). As neointima starts to form, the artery can prevent loss of lumen volume by dilatation, a phenomenon called remodeling, which to some extent can compensate for the increase in neointimal mass (Waller et al. 1991, Mintz et al. 1996). However, if the process of vascular inflammation continues, it triggers a positive feedback mechanism, causing further accumulation of mononuclear cells and the migration and proliferation of SMCs at the site of injury. Eventually the arterial lumen will be critically diminished and blood flow subsequently compromised (Ross 1999).

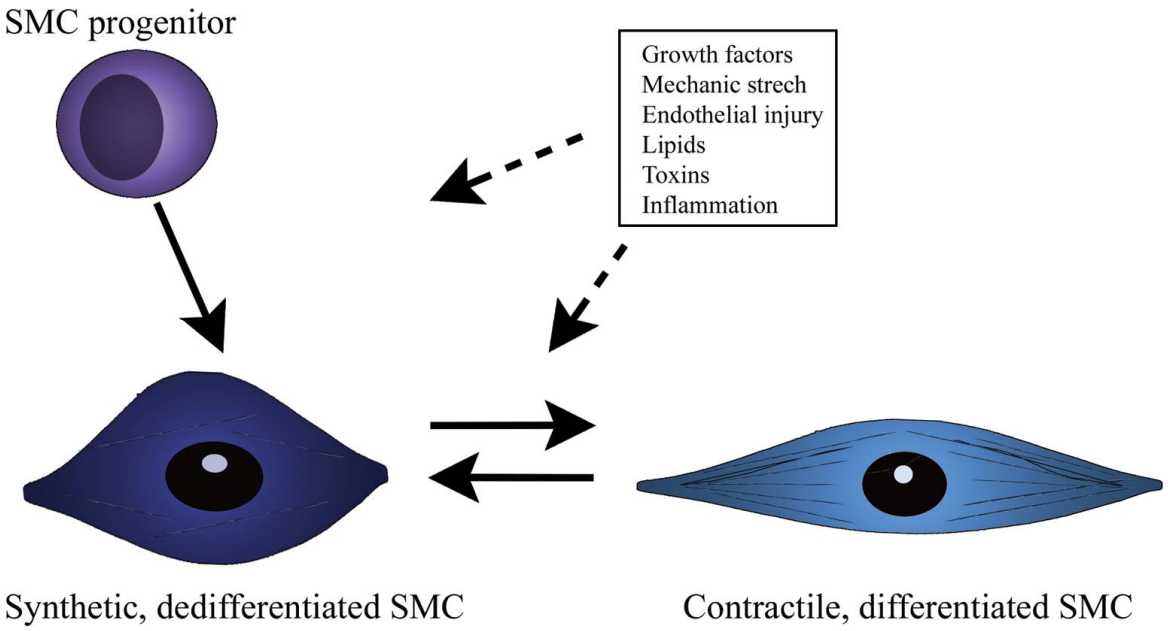

Figure 2 *Smooth muscle cell phenotypic switch is a hallmark feature of neointimal development.*

2.3 Vascular smooth muscle cells

Smooth muscle cells play a pivotal role in vessel wall biology and the development of vascular disease, including atherosclerosis, restenosis, and transplant arteriosclerosis. SMCs are not terminally differentiated, and can change their phenotype upon local environmental cues. Following vascular injury, SMCs accumulate into the intima, migrate, proliferate, and synthesize extracellular matrix (Holmes et al. 1984). Neointimal SMCs differ in phenotype from medial SMCs, and they are described as dedifferentiated and immature cells of a more synthetic phenotype. The synthetic SMCs are characterized by their lower levels of SMC contractile proteins and fewer myofilaments, higher synthetical activity, and the expression of a large number of proteins that may contribute to the development of the neointima (Schwartz et al. 1986, Campbell et al. 1990) (Fig. 2).

The origin of neointimal SMCs has evoked much controversy. It has long been assumed that SMCs migrate to the neointima from the media (Thyberg et al. 1990), and that either all medial SMCs (Ross 1999), or only a certain subpopulation of these cells (Benditt et al. 1973) are capable of phenotypic modulation. After the neointimal SMC population was shown to be heterogenic (Orlandi et al. 1994, Schwartz et al. 1998), neointimal cells have been suggested to originate from adventitial fibroblasts that transform into myofibroblasts (Shi et al. 1996b, Li et al. 2000), or endothelial cells (Gittenberger-de Groot et al. 1999). It has also been proposed that neointimal SMCs derive from a subpopulation of immature cells present in the media or adventitia (Majesky et al. 1992, Holifield et al. 1996).

These views have been challenged by reports indicating circulating progenitor cells as a potential source of neointimal SMCs (Saiura et al. 2001). In allograft experiments, the cells migrating into the vascular intima during chronic rejection in mice, rats, and humans are derived from the recipient, not the donor (Hillebrands et al. 2001, Grimm et al. 2001). In rodent models using green fluorescent protein or LacZ labeling, or sex-mismatched cells, bone marrow stem cells have been shown to contribute to neointimal formation in restenosis, allograft arteriosclerosis, and hyperlipidemia-induced atherosclerosis (Campbell et al. 2001, Han et al. 2001, Shimizu et al. 2001, Sata et al. 2002, Matsumoto et al. 2003, Xu et al. 2004). These results are supported by the notion that adult bone marrow contains multipotent cells that can develop into various cell lineages (Pittenger et al. 1999), and that endothelial progenitor cells can transdifferentiate into SMCs (DeRuiter et al. 1997). The proposed origins of neointimal SMCs are summarized in Figure 3.

However, contradictory evidence also exist regarding the bone marrow origin of the circulating precursors (Li et al. 2001, Hillebrands et al. 2002, Hu et al. 2002a, Hu et al. 2002b). Also the suggested participation rate of these bone marrow precursor cells, as well as the percentage of neointimal cells of perivascular origin, vary significantly between different reports (Table 2). These discrepancies have been suggested to arise from the diversity of neointimal cell origin, and from differences in the models used (Tanaka et al. 2003). It is also possible that stem cells adopt different mature cell phenotypes by cell fusion instead of transdifferentiation (Terada et al. 2002, Ying et al. 2002, Vassilopoulos et al. 2003).

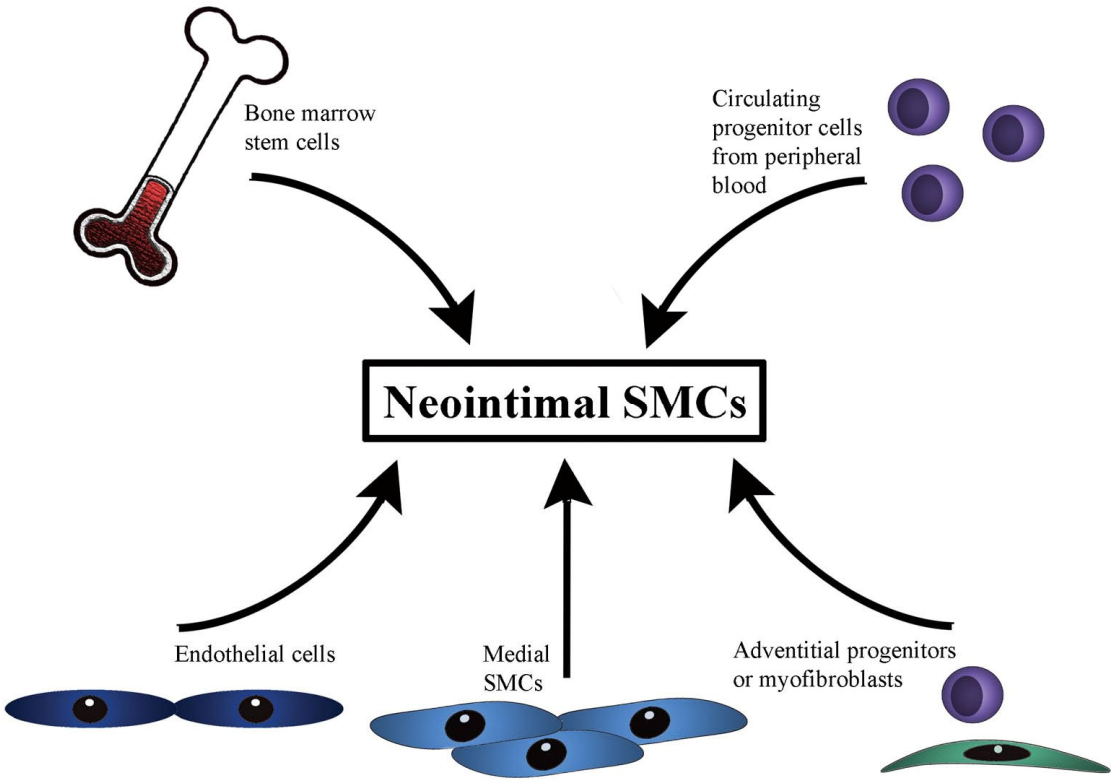

Figure 3 *Potential origins of neointimal SMCs.*

Most data on the progenitor origin of neointimal cells derives from animal experiments, and it is unclear how well these results can be applied to humans. In animal models it has been suggested that bone marrow cells are recruited only when severe medial damage occurs (Campbell et al. 2001, Tanaka et al. 2003), and this kind of injury should be quite uncommon in the clinic. Nevertheless, human peripheral blood contains CD34+ and Flk-1+ precursors capable of differentiating to SMCs (Simper et al. 2002). SMCs in coronary atherosclerosis have been shown to be of bone marrow origin (Caplice et al. 2003) and vascular progenitor cells are known to exist in human coronary in-stent restenosis (Hibbert et al. 2004) and atherosclerotic lesions (Torsney et al. 2007). Intracoronary bone marrow cell infusion has evoked interest as a way of improving cardiac function after coronary stenting in myocardial infarction. However, in clinical trials this approach has been complicated by accelerated atherosclerosis (Kang et al. 2004, Mansour et al. 2006).

Table 2 *Proposed participation rate of progenitor cells of perivascular origin in neointimal hyperplasia in various mouse arterial injury models.*

Model/artery	**Progenitor cells in neointima**	**Progenitor cells in media**	**Days after injury**	**Reference**
Wire-induced injury				
iliac artery	56%	n.d.	28	Campbell et al. 2001
femoral artery	56%	n.d.	28	Han et al. 2001
femoral artery	63%	46%	7	Sata et al. 2002
femoral artery	56%	54%	28	Tanaka et al. 2003
femoral artery*	39%	61%	28	Tanaka et al. 2003
Cuff-induced injury				
femoral artery	"majority"	0%	7 and 14	Xu et al. 2004
femoral artery	"seldom"	0%	28	Tanaka et al. 2003
femoral artery*	7%	15%	28	Tanaka et al. 2003
Ligation-induced injury				
common carotid artery	"a few"	0%	28	Tanaka et al. 2003
common carotid artery*	24%	33%	28	Tanaka et al. 2003

* ApoE-deficient mice

3. Potential vasculoprotective compounds

Potential treatments for vasculoproliferative disorders have been studied extensively. Still, no definite cure for atherosclerosis, restenosis, or transplant arteriopathy has emerged. This section will review compounds that have shown vasculoprotective properties in this thesis study.

3.1 Somatostatin and somatostatin analogs

3.1.1 Introduction to natural somatostatin

Somatostatin was first described in the late 1960s by Krulich and coworkers as well as by Hellman and Lernmark separately (Krulich et al. 1968, Hellman and Lernmark 1969). A few years later somatostatin was isolated from the hypothalamus (Brazeau et al. 1973). This neurohormone, produced by neural, endocrine, and exocrine cells, acts both systemically as a true hormone, and locally as a neurotransmitter inhibiting cellular proliferation, and modulating hormone and growth factor secretion (Reichlin 1983, Mascardo and Sherline 1982, Payan et al. 1984, Patel et al. 1999). The classical effects of

somatostatin include inhibition of pituitary gland growth hormone and thyroid-stimulating hormone secretion (Brazeau et al. 1973, Reichlin 1983), inhibition of insulin and glucagon release from the Islets of Langerhans (Vaysse et al. 1981, Reichlin 1983), and release of gastrointestinal hormones from the stomach, gut, and the pancreas (Bloom et al. 1974, 1975). Somatostatin is also involved as a neuromodulator in the central nervous system (Epelbaum et al. 1986, Gillies 1997), and as a vasoconstrictor in the gastrointestinal system (Sieber et al. 1992).

The natural forms of somatostatin, harvested by splicing from prosomatostatin (Hobart et al. 1980, Goodman et al. 1980), are somatostatin-14 (SST-14) with equal affinity for all somatostatin receptors (Patel and Srikant 1994), and somatostatin-28 (SST-28) with a slightly higher affinity for sst_5 (Reisine and Bell 1995). Cortistatin is a more recently discovered natural somatostatin analog with nanomolar affinity for all somatostatin receptors (Fukusumi et al. 1997, Patel et al. 1997).

Table 3 *Properties of human and rat somatostatin receptors.*

Property	sst_1	sst_2	sst_3	sst_4	sst_5	Reference
Chromosomal location (human)	14q13	17q24	22q13.1	20p11.2	16p13.3	Corness et al. 1993 Demchyshyn et al. 1993 Yamada et al. 1993 Panetta et al. 1994
Chromosomal location (rat)	6q23	10q32.1	7q34	3q41	10q12	Bruno et al. 1992 Li et al. 1992 Kluxen et al. 1992 Meyerhof et al. 1992 O'Carroll et al. 1992
Receptor identity rat vs. human	94%	94%	83%	88%	82%	Patel et al. 1995

3.1.2 The somatostatin receptor families

The 7-transmembrane G-protein coupled somatostatin receptors (sst) sst_1, sst_{2A}, sst_{2B}, sst_3, sst_4, and sst_5, identified in the early 1990s (Yamada et al. 1992a, 1992b, Rohrer et al. 1993, Panetta et al. 1994), are encoded by five separate genes, all located on different chromosomes (Corness et al. 1993, Demchyshyn et al. 1993, Panetta et al. 1994, Yamada et al. 1993) (Table 3). The sst_{2A} and sst_{2B} are thought to arise from alternative splicing of the sst_2 mRNA (Patel et al. 1993). Based on their structure and functions, the receptors have been divided into two families, the $sst_2/sst_3/sst_5$ and the sst_1/sst_4 family (Hoyer et al. 1995, Patel et al. 1995, Reisine and Bell 1995). The homology between rat and human somatostatin receptors is high, ranging from 82% (sst_5) to 94% (sst_1), but there are significant species specific variations in sst functions (Patel et al. 1995, Olias et al. 2004).

The development of somatostatin receptor agonists and antagonists (Patel and Srikant 1994, Bass et al. 1996), as well as antibodies for each somatostatin receptor subtype (Schulz et al. 2000) has provided more information about the tissue-distribution and function of the individual somatostatin receptors. The sst_2 is expressed in pancreatic α-cells (Hunyady et al. 1997) and mediates the inhibition of glucagon release (Rohrer et al. 1998, Strowski et al. 2000), while sst_5 is localized in the insulin-secreting pancreatic β-cells (Mitra et al. 1999), and mediates the inhibition of insulin release (Rohrer et al. 1998, Strowski et al. 2000). Both receptor subtypes co-localize in pituitary gland somatotrophs and mediate the inhibition of growth hormone release (Mezey et al. 1998, Rohrer et al. 1998, Patel 1999). The other receptor subtypes are believed not to be involved in mediating these classical somatostatin effects (Rohrer et al. 1998).

The role of somatostatin in modulating the immune system has evoked much interest. In humans, sst_2 has been proposed to modulate the function of inflammatory cells (Patel 1999), and somatostatin acts as a chemoattractant to sst_2-expressing human bone marrow and peripheral blood-derived CD34+ cells *in vitro* (Oomen et al. 2002, Lichtenauer-Kaligis et al. 2004). In the rat immune system, sst_3 and sst_4 predominate, while sst_2 is completely absent (ten Bokum et al. 1999), and targeting through sst_4 inhibits a variety of inflammatory processes (Helyes et al. 2006).

3.1.3 Somatostatin receptor signaling

All somatostatin receptors mediate their effects through inhibition of adenylyl cyclase, depression of intracellular cAMP levels (Hoyer et al. 1994, Patel et al. 1994), and modulation of mitogen-activated protein kinase (MAPK) (Bito et al. 1994, Cordelier et al. 1997, Yoshitomi et al. 1997, Florio et al. 1999, Cattaneo et al. 2000). Somatostatin receptors also activate phosphotyrosine phosphatase (Florio et al 1994, Reardon et al. 1997, Sharma et al. 1999), modulate K^+ ion channels (Karschin 1995, Kreienkamp et al. 1997), and couple to phospholipase C (Akbar et al. 1994, Murthy et al. 1996, Lee et al. 1998, Siehler and Hoyer 1999, Cervia et al. 2003) (Fig. 4). Furthermore, some receptor subtypes modulate voltage-dependent Ca^{2+} ion channels (Fujii et al. 1994, Tallent et al. 1996, Roosterman et al. 1998), Na^+/H^+ exchangers (Hou et al. 1994, Schindler et al. 1998, Smalley et al. 1998, Chen and Tashjian 1999), and AMPA/kainate glutamate channels (Lanneau et al. 1998).

The expression of somatostatin receptors in different cell types frequently overlap, and a single cell can co-express several or even all somatostatin receptor subtypes, suggesting that the effects of somatostatin usually are mediated through several ssts working together (Patel 1999). *In vitro*, at least some of the somatostatin receptors are known to undergo homodimerization with receptors of the same subtype, or heterodimerization with other ssts (Rocheville et al. 2000a, Pfeiffer et al. 2001), or related GPCRs, as opioid (Pfeiffer et al. 2002) and dopamine receptors (Rocheville et al. 2000b). Several functional interactions between somatostatin receptors have also been reported, such as interactions between sst_{2A} and sst_5 (Cervia et al. 2003, Sharif et al. 2007), sst_2 and sst_4 (Moneta et al. 2002), and sst_1 and sst_2 (Pavan et al. 2004). When applicable also to *in vivo* situations, these phenomena

can alter ligand binding affinities, function and regulation of the receptors involved, further enhancing the functional diversity of somatostatin receptors (Ferone et al. 2007).

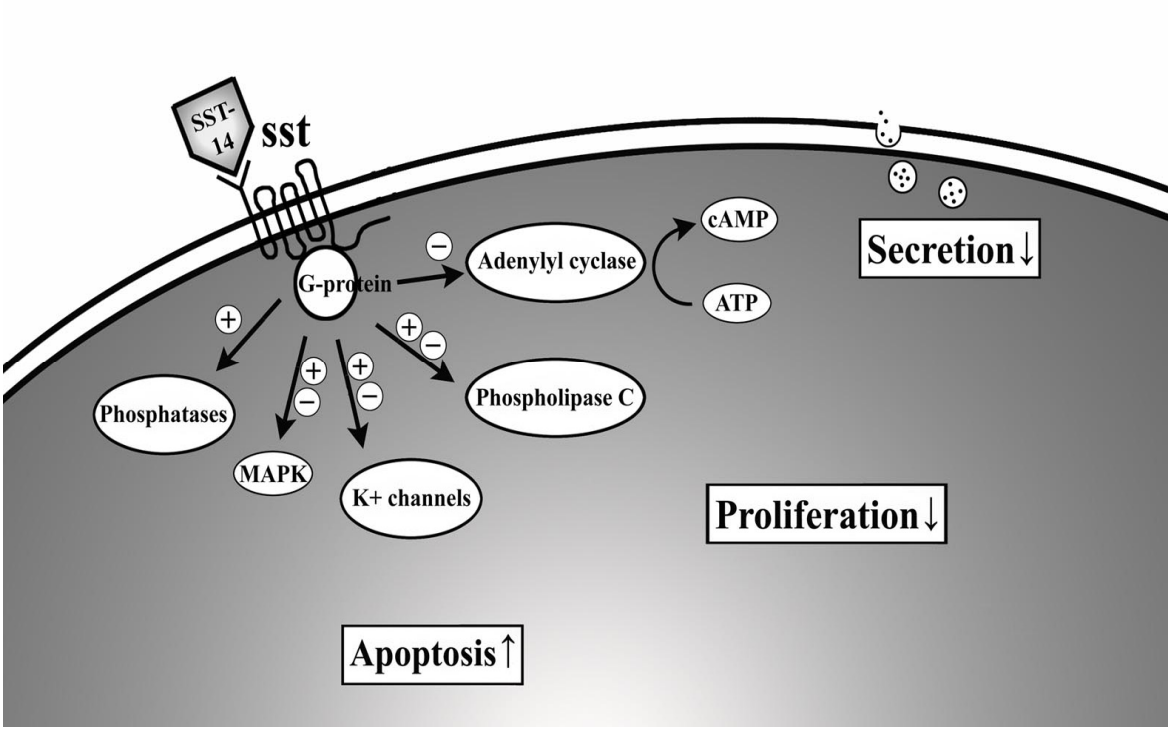

Figure 4 *A schematic illustration of the coupling of somatostatin receptors to different signaling pathways leading to inhibition of secretion and cell proliferation, and stimulation of cell apoptosis. MAPK, mitogen-activated protein kinase.*

3.1.4 Discovery and characterization of synthetic somatostatin analogs

The use of somatostatin in clinical practice has been limited by its short half-life, <3 min, and lack of selectivity (Janecka et al. 2001). Thus, numerous somatostatin analogs with improved pharmacokinetics and with different receptor subtype selectivity profiles have been developed.

Octreotide (SMS 201-995), the first long-acting synthetic octapeptide somatostatin analog developed into clinical use, exerts its action by binding to sst_2, sst_3, and sst_5, thus reducing the secretion of insulin, glucagon, vasoactive intestinal peptide, gastric acid, thyroid-stimulating hormone, and growth hormone (Bauer et al. 1982, Lamberts et al. 1996). Lanreotide (somatuline, angiopeptin, BIM-23014) also shows selectivity for sst_2, sst_3, and sst_5, and has actions similar to octreotide. These analogs are in clinical use for the treatment of conditions such as acromegaly, for the prevention of complications after

pancreatic surgery and esophageal variceal bleedings, and for the symptomatic treatment of some hormone producing tumours.

CH275, the first somatostatin receptor analog targeting the sst_1/sst_4 receptor family, was discovered in the late 1990's (Liapakis et al. 1996, Patel 1997). Soon thereafter, non-peptide somatostatin receptor subtype specific agonists for all receptor subtypes were described (Rohrer et al. 1998). Non-peptide agonists have evoked much interest due to the possibility of developing oral somatostatin therapies.

3.1.5 Vascular expression of somatostatin receptors

The reported sst subtype expression in rat and human vascular tissue and cells is summarized in Table 4. In rat, all somatostatin receptor subtypes are expressed in vascular tissue, but sst_2 and sst_5 are expressed at much lower levels than the other receptor subtypes (Khare et al. 1999). The expression of sst_1 peaks acutely after endothelial injury, whereas the expression of sst_3 and sst_4 starts to increase more slowly and remains elevated during vessel repair (Khare et al. 1999). In uninjured rat vessels, the somatostatin receptors are mostly expressed in the media. After endothelial injury, the expression of sst_1, sst_3, and sst_4 shifts to neointimal SMCs (Khare et al. 1999). Adventitial cells do not express any somatostatin receptors (Khare et al. 1999).

In normal and diseased human arteries, the predominant receptor subtypes are sst_1 and sst_4, with sst_2 expressed only at low levels (Curtis et al. 2000). The expression of sst_3 and sst_5 is absent (Curtis et al. 2000). The endothelium of atherosclerotic arteries has been shown to express mostly sst_1 (Curtis et al. 2000), while results from studies of sst expression in cultured endothelial cells show significant variation. Human umbilical vein endothelial cells (HUVECs), have been shown to express sst_1, sst_2, and sst_5 (Adams et al. 2004, 2005), sst_3 only (Florio et al. 2003, Jia et al. 2003), or sst_1 and sst_4 (Curtis et al. 2000). In cultured human coronary endothelial cells the reported sst expression ranges from the expression of sst_4 only (Badway et al. 2004) to the expression of sst_1, sst_2, and sst_5 (Yan et al. 2005).

Cultured human vascular SMCs express sst_1 and sst_2 (Curtis et al. 2000), while rat SMCs express sst_4 only (Torrecillas et al. 1999).

Table 4 *Reported expression of somatostatin receptors in vascular tissue and cells.*

Tissue/Cell type	sst_1	sst_2	sst_3	sst_4	sst_5	Reference
Human vascular tissue						
normal artery	+	+	0	+	0	Curtis et al. 2000
injured artery	+	+	0	+	0	Curtis et al. 2000
Rat vascular tissue						
normal artery	+	+	+	+	+-	Reynaert et al. 2007
normal artery	+	+-	+	+	+-	Khare et al. 1999
normal artery	n.d.	+	0	n.d.	0	Chen et al. 1997a
injured artery	+	+	+	+	+	Khare et al. 1999
injured artery	n.d.	+	0	n.d.	0	Chen et al. 1997a
Human endothelial cells						
HUVECs	+	0	0	+	0	Curtis et al. 2000
HUVECs	+	+	0	0	+	Adams et al. 2004
HUVECs	0	0	+	0	0	Jia et al. 2003
proliferating HUVECs	+	+	0	0	+	Adams et al. 2005
Eahy926*	0	0	+	0	0	Florio et al. 2003
ECV304**	+	0	0	+	0	Curtis et al. 2000
hCAEC***	0	0	0	+	0	Badway et al. 2004
hCAEC***	+	+	0	0	+	Yan et al. 2005
Human SMCs						
human aortic SMCs	+	+	0	0	0	Curtis et al. 2000
Rat SMCs						
primary rat aortic SMCs	0	0	0	+	0	Torrecillas et al. 1999

* cell line derived from the fusion of HUVECs with the A549 cell line; ** transformed HUVEC cell line; *** human coronary artery endothelial cells; n.d., not defined; 0 = no expression; +- = barely detectable; + = clearly expressed

3.1.6 Vascular effects of somatostatin and its analogs

In experimental models, somatostatin and its analogs are known to inhibit SMC proliferation and migration (Häyry et al. 1993b, Grant et al. 1994, Mooradian et al. 1995, Lauder et al. 1997), as well as proliferation of endothelial cells (Lawnicka et al. 2000, Adams et al. 2004, 2005). In addition, somatostatin inhibits endothelial cell adhesion molecule expression (Badway et al. 2004).

In 1989, Lundergan and coworkers showed that lanreotide, selective for $sst_2/sst_3/sst_5$, and the related compound BIM23034 inhibited intimal hyperplasia after rat carotid artery injury (Lundergan et al. 1989). The results were repeated in different rodent (Lundegan et al. 1991, Mennander et al. 1993, Häyry et al. 1993b, Takahashi et al. 1995, Zhao et al. 1997), rabbit (Howell et al. 1993, Bauters et al. 1994, Foegh et al. 1994) and porcine models (Santoian et al. 1993, Hong et al. 1997). An inhibitory effect on intimal

hyperplasia after denudation injury has also been reported for octreotide (Yumi et al. 1997, Yamashita et al. 1999). Furthermore, both compounds have been demonstrated to prevent graft vessel disease in rat (Häyry et al. 1993b, Mennander et al. 1993, Bruns et al. 2000).

Despite the promising preclinical results, three multicenter trials for the prevention of reocclusion or clinical events after PTCA could show only a marginal effect with lanreotide (Emmanuelsson et al. 1995, Eriksen et al. 1995) and none with octreotide (von Essen et al. 1997). Local drug delivery at the site of injury has been successful with lanreotide in a rabbit model (Hong et al. 1993), but not in pigs (Armstrong et al. 2002). These results have diminished the enthusiasm for developing somatostatin analogs with vasculoprotective properties. Recently, promising results were obtained in a pilot study of 14 patients treated with a lanreotide-eluting stent (Kwok et al. 2005). Still, no somatostatin analogs are currently used routinely in the clinic for the treatment of vasculoproliferative disorders.

The mechanisms behind the potential vasculoprotective effects of somatostatin are not fully understood. Somatostatin and sst_2/sst_3/sst_5 analogs have been proposed to interfere with SMC proliferation through inhibition of growth factors, such as insulin-like growth factor-1 (IGF-1), bFGF, and PDGF-BB (Häyry et al. 1993b, Mennander et al. 1993, Grant et al. 1994, Lauder et al. 1997). It has also been suggested that sst_2/sst_3/sst_5 analogs affect autocrine and paracrine mechanisms that regulate cell replication (Lundergan et al. 1991), or endocrine factors such as growth hormone release (Tiell et al. 1978, Bruns et al. 2000). A novel theory is that the vasculoprotective effects of somatostatin are due to anti-inflammatory effects on the vascular endothelium (Badway et al. 2004, Yan et al. 2005).

3.2 Sirolimus

3.2.1 Discovery and characterization

Sirolimus (rapamycin, AY-22,989, Rapamune®) was isolated from a streptomycete found in soil samples during a systematical search for new antibiotics (Vezina et al. 1975). The samples originated from the Easter Islands, also known as Rapa Nui, which is why the compound initially got the name rapamycin. Rapamycin, by generic name sirolimus, was originally characterized as an antifungal antibiotic (Sehgal et al. 1975, Vezina et al. 1975). The immunosuppressive effects of sirolimus were observed early (Martel et al. 1977), and its development as an antifungal drug was stopped. In 1989, the first report was published stating that sirolimus could be used for immunosuppression in organ allografting (Calne et al. 1989). Almost a decade later the drug entered clinical trials and it proved to be both safe and effective as an immunosuppressant after kidney transplantation (Murgia et al. 1996, Kahan et al. 1998).

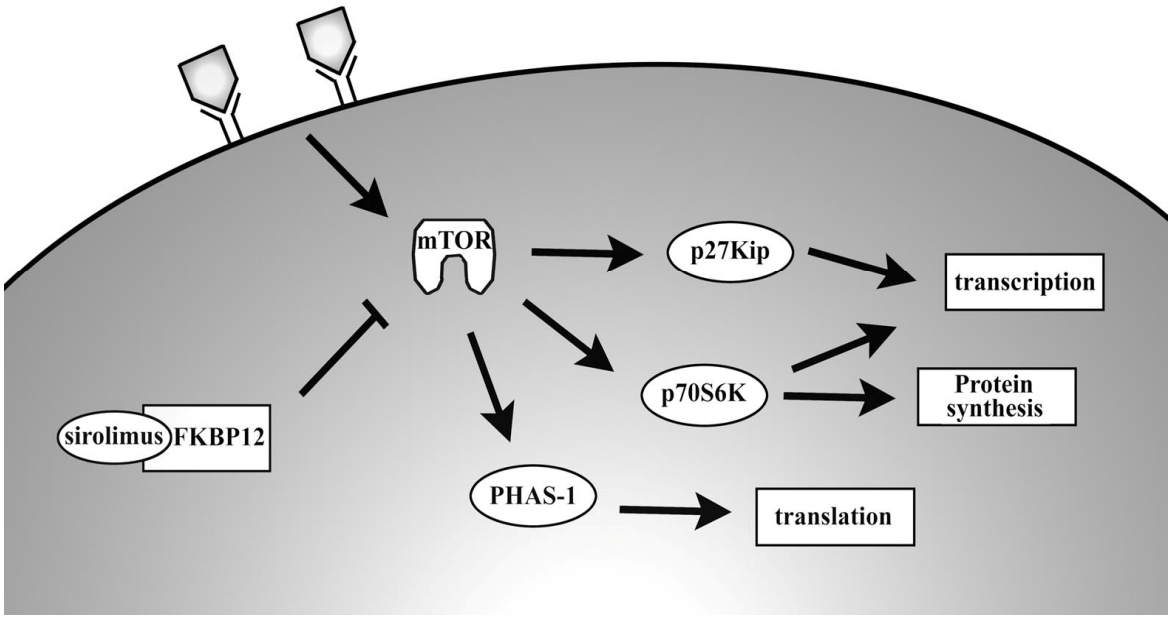

Figure 5 *The sirolimus:FKBP12 complex inhibits growth factor signaling and cell cycle progression through inhibition of mTOR.*

3.2.2 Mechanisms of action and clinical pharmacology

Sirolimus binds to the family of intracellular receptors termed FK binding proteins (FKBPs) (Brown et al. 1994, Chen et al. 1994), of which FKBP12 is the most relevant in mediating the effects of sirolimus (Jayaraman et al. 1992). The sirolimus:FKBP complex is thought to initiate most of its actions through inhibition of mTOR (Chiu et al. 1994, Sabers et al. 1995). The inhibition of mTOR modulates several intracellular pathways by inhibiting proteins such as the p70S6 kinase (Chung et al. 1992, Price et al. 1992), cycline kinase inhibitor p27Kip (Nourse et al. 1994), and phosphorylatable heat stable protein (PHAS-1) (Beretta et al. 1996, Brown et al. 1996, Brunn et al. 1997). (Fig. 5). The effects on intracellular pathways finally disrupt cell cycle progression from the G1 to the S phase, thus inhibiting cell proliferation (Flanagan et al. 1993, Terada et al. 1993). The immunosuppressive effect of sirolimus is mediated through inhibition of T cells (Flanagan et al. 1993, Terada et al. 1993) and B cells (Aaguaard-Tillery et al. 1994, Kim et al. 1994).

Sirolimus is a rapidly absorbed drug (maximum blood concentration at 1 hour) with a low systemic availability (14%) (Brattstrom et al. 2000, MacDonald et al. 2000). The half-life of sirolimus is long, 62 hours (Zimmerman and Kahan 1997), and the 2.5-fold accumulation of sirolimus blood concentrations for 6 days suggests the need for a loading dose that is 3 times higher than the maintenance dose (Zimmerman and Kahan 1997). Sirolimus is metabolized by cytochrome P450 (CYP) 3A4 (Sattler et al. 1992, Lampen et

al. 1998) and P-glycoprotein (Saeki et al. 1993). The side-effects most commonly associated with sirolimus treatment are a decrease in platelet and white blood cell counts and hemoglobin, and increased cholesterol values (Murgia et al. 1996, Brara et al. 2003).

3.2.3 Vascular effects of sirolimus

In animal experiments oral sirolimus has been effective in preventing intimal hyperplasia after ballooning injury, and the side-effects have been minor (Gregory et al. 1993a, Burke et al. 1999, Gallo et al. 1999). Treatment with oral sirolimus after implantation of a bare metal stent has been shown to reduce restenosis rates (Rodriguez et al. 2003, Waksman et al. 2004, Hausleiter et al. 2004, Rodriguez et al. 2006), although contradictory evidence also exists (Brara et al. 2003). However, systemic sirolimus treatment has been associated with side-effects in more than 50% of the patients (Brara et al. 2003, Waksman et al. 2004).

Local therapy with sirolimus-coated intracoronary stents has evoked much interest. The first reports on sirolimus-eluting stents in low-risk patients with short *de novo* lesions showed how the application of the stent virtually abolished restenosis (Rensing et al. 2001, Sousa et al. 2001a, 2001b). The first larger trials confirmed the benefits of SESs, although the results were not quite as impressive as in the pilot studies (Morice et al. 2002, Moses et al. 2003).

Some studies have supported the efficacy of SESs also in high risk patients (Moses et al. 2003, Holmes et al. 2004), and in patients with lesions in the left main coronary artery (Arampatzis et al. 2003), long *de novo* lesions in small coronary arteries (Schampaert et al. 2004), and in patients with in-stent restenosis (Sousa et al. 2003). More recently, these more and more common "off-label" indications of SESs have evoked safety concerns, and they may associate with a higher risk of death and myocardial infarction (Pfisterer et al. 2006, Lagerqvist et al. 2007). Furthermore, SESs have been reported to increase the risk of often fatal stent thrombosis (McFadden et al. 2004, Iakovou et al. 2005, Bavry et al. 2006), and hypersensitivity reactions (Virmani et al. 2004). Characteristic for thrombosis associated with drug-eluting stents has been the emergence of very late thrombosis (> 12 months after PTCA), occuring even later than three years post-PTCA (Daemen et al. 2007).

The effects of sirolimus on neointimal hyperplasia are believed to arise from inhibition of SMC proliferation and migration (Gregory et al. 1993b, Cao et al. 1995, Marx et al. 1995, Poon et al. 1996) through modulation of the cycline-dependent kinase p27Kip1 (Cao et al. 1995, Gallo et al. 1999, Sun et al. 2001). Sirolimus may also exert inhibitory effects on circulating smooth muscle progenitor cells and inhibit their differentiation to neointimal SMCs (Fukuda et al. 2005). However, sirolimus also suppresses endothelial cell proliferation (Akselband et al. 1991) by blocking the p70S6 kinase pathway (Vinals et al. 1999), and modulating endothelial progenitor cell proliferation and differentiation (Butzal et al. 2004, Chen et al. 2006). These effects might explain the impaired re-endothelialization associated with SESs (Joner et al. 2006, Luscher et al. 2007).

3.3 Imatinib

3.3.1 Discovery and characterization

Imatinib mesylate (STI-571, CGP 57148B, Glivec®, Gleevec®) is a protein-tyrosine kinase (PTK) inhibitor that was found during a large screening for Abl PTK inhibitors (Druker et al. 2000). The most well-known indication for imatinib treatment is chronic myelogenous leukemia, characterized by the Bcr-Abl PTK oncoprotein (Konopka et al. 1984, Daley et al. 1990), which is the target of imatinib treatment. Imatinib is also used in the treatment of gastrointestinal stromal tumours (van Oosterom et al. 2001), characterized by mutations of c-kit, and occasionally also the platelet-derived growth factor receptor (PDGF-R) (Hirota et al. 1998). More recently other indications for imatinib treatment have emerged, such as Philadelphia chromosome-positive acute lymphoblastic leukemia, and myelodysplastic diseases.

3.3.2 Mechanisms of action and clinical pharmacology

PTKs belong to the family of protein kinase enzymes, and they are parts of signaling cascades controlling cell growth, adhesion, metabolism, differentiation and apoptosis (Robinson et al. 2000). Imatinib inhibits the Abl (Buchdunger et al. 1996, Druker et al. 1996, Carroll et al. 1997), ARG (Okuda et al. 2001), PDGF-R-α and β (Carroll et al. 1997, Buchdunger et al. 2000), and c-kit (Buchdunger et al. 2000, Heinrich et al. 2000) tyrosine kinases. However, imatinib has no effect on closely related kinases such as c-Fms, Kdr, Flt-1, Tek, and Flt-3 (Buchdunger et al. 2000), v-Fms, c-erbB1, c-erbB2, insulin receptor, IGF-1 receptor, Jak-2, and v-Src (Buchdunger et al. 2002).

Imatinib has an excellent oral bioavailability, and in general treatment is well tolerated (Druker et al. 2001a, 2001b). Metabolization of imatinib occurs mainly by the CYP3A4 or CYP3A5 (Peng et al. 2005), and it has a half-life of 12-14 hours (Druker et al. 2000). The most common side-effects of imatinib treatment are nausea, vomiting, oedema and muscle cramps; rare side-effects include liver toxicity, fluid-retention, neutropenia, and thromocytopenia (Kantarjian et al. 2002, Sawyers et al. 2002, Talpaz et al. 2002).

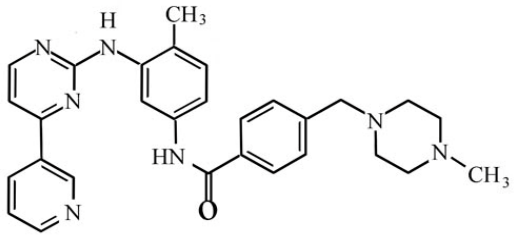

Figure 6 *Molecular structure of imatinib.*

3.3.3 Vascular effects of imatinib

Because of its inhibitory effects on PDGF-R, imatinib has also evoked interest as a potential vasculoprotective compound. In *in vitro* settings, imatinib inhibits human and rodent vascular SMC proliferation and migration (Myllärniemi et al. 1999, Dudley et al. 2003, Hacker et al. 2007), without affecting endothelial cell proliferation (Gambacorti-Passerini et al. 1997, Hacker et al. 2007). In *in vivo* rodent models, imatinib has been shown to inhibit denudation injury-induced neointimal hyperplasia (Myllärniemi et al. 1999, Wang et al. 2006) as well as transplant arteriopathy (Sihvola et al. 2003), and diabetes-associated atherosclerosis (Lassila et al. 2004).

However, in a standard porcine coronary overstretch model with localized drug delivery, imatinib did not prevent neointimal proliferation (Hacker et al. 2007). Also, in a pilot clinical trial imatinib failed to prevent recurrent restenosis in patients receiving the drug for 2 days prior to, and 7 days after operation (Zohlnhofer et al. 2005). Therefore, combination therapies have raised interest, and the addition of sirolimus (Vamvakopoulos et al. 2006), or vascular endothelial growth factor-C gene transfer to the treatment protocol (Leppänen et al. 2004) has enhanced the vasculoprotective properties of imatinib.

The vascular effects of imatinib have been thought to arise from its inhibitory effects on PDGF-R-dependent processes, such as SMC activation and macrophage infiltration (Myllärniemi et al. 1999, Lassila et al. 2004, Leppänen et al. 2004, Hacker et al. 2007). Also, c-kit and its ligand stem cell factor (SCF) (Chabot et al. 1988) have been proposed as essential in the development of neointimal hyperplasia (Hollenbeck et al. 2004), and it has been suggested that imatinib could reduce intimal hyperplasia by modulating vascular progenitor cell activity through inhibition of c-kit (Wang et al. 2006). The role of c-Abl inhibition has not been determined, but c-Abl is a downstream mediator of PDGF signaling (Plattner et al. 2003), and could thus also be a target of imatinib treatment in vasculoproliferative disorders.

AIMS OF THE STUDY

The aim of this study was to gain knowledge about the mechanisms of arterial injury repair and especially the post-injury mobilization of precursor cells to the vessel wall and their differentiation into SMCs that contribute to neointimal hyperplasia. This knowledge is essential in the search for novel drug candidates for the treatment of vasculoproliferative disorders, and for understanding how these drugs affect the cells in the vascular wall.

The specific aims of the study were:

1. To set up an *ex vivo* model for neointimal hyperplasia that reflects remodeling after intimal injury closer than *in vitro* SMC culture studies or medial explants and also takes into account the participation of precursor cells.

2. To investigate the roles of the five somatostatin receptors in neointima formation after denudation injury, and to determine the vasculoprotective properties of sst-agonists and their effects on neointimal cell migration and proliferation.

3. To investigate the mechanisms behind the synergistic effect of the sirolimus-imatinib combination in inhibiting neointimal hyperplasia, as well as the long-term efficacy of the treatment.

4. To identify possible targets for the development of vasculoprotective therapies.

METHODS

1. Rat models of restenosis

Experimental animals (I-IV)

Male Wistar rats (250 - 350 g) were purchased from the Laboratory Animal Center, University of Helsinki, Finland (II) or from Harlan, Horst, Holland (I, III, IV). The studies were approved by the Haartman Institue Ethical Committee for Animal Studies, and the permit for animal studies was granted by the Government of the County of Southern Finland. Laboratory rats were treated according to the Finnish law on animal rights (9§ 777/85). All animals received humane care in compliance with the European agreement for the use of experimental animals in scientific research, and with the principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, prepared and formulated by the National Institute of Laboratory Animal Resources, published by the National Institutes of Health (NIH publication no. 85-23, revised 1996). The basic diet of the animals was pellets (Altromin N:o 1314, Standard diet, Chr. Petersen A/S, Ringsted, Denmark) and they were given tap water *ad libitum*. The rats were anesthetized with chloral hydrate (240 mg/kg i.p.) and peri- and postoperative pain was treated with buprenorphine (0.1 mg/kg s.c., Reckitt & Coleman, Hull, England). The permit to use chloral hydrate anesthesia was received from the County veterinarian.

Rat carotid artery and aortic denudation (I-IV)

The carotid denudation injury was performed by introducing a 2-French Fogarty balloon embolectomy catheter (Baxter Healthcare Corp, Santa Ana, CA) into the common carotid artery through the left external carotid artery and inflating the catheter with 0.2 ml of air. The inflated catheter resulted in a 0.5 lbs pull force and a balloon size of 4 mm, and the catheter was retrieved three times. After removal of the catheter, the external carotid artery was ligated and the wound was closed. In the aortic artery denudation model, the embolectomy catheter was introduced into the thoracic aorta via the left iliac artery, inflated with 0.2 ml air, and passed five times to remove the endothelium. Thereafter, the iliac artery was ligated and the wound was closed. Upon sacrifice, the denuded artery was removed, and the mid-section of the artery was processed for histology. The rest of the artery was either immediately processed for explant cultures, or frozen in liquid nitrogen and stored at -70ºC for later RNA isolation.

2. Drug administration and dosages

Somatostatin and somatostatin analogs (II, III, and unpublished results)

SST-14 (NovaBiochem/Sigma, St. Louis, MO), CH275, and octreotide (NeoSystems Strasbourg, France) were diluted in PBS and administered as daily s.c. injections (50-500 µg/kg), or SST-14 and CH275 by using 7-day or 14-day osmotic minipumps (Alzet, Palo Alto, CA) at constant flow rate at equivalent dose levels. The drug treatment was initiated at the operation, and control rats received vehicle only. The C18-HPLC profile provided by the suppliers showed that the peptides were highly homogenous: SST-14 >99.5%, CH275 97%, and octreotide 98.1%. For *in vitro* studies, the compounds were diluted in PBS, and a 25 mM stock solution was prepared. In the *in vitro* cell line studies, drug concentrations of 0.1, 1, 5, and 10 µM were used; in the *ex vivo* explant studies the drug concentrations were 500 µg/kg *in vivo* and 10 µM *in vitro*.

The somatostatin receptor subtype selective agonists were a kind gift from Dr. Susan P. Rohrer, Merck & Co., Inc., Rahway, NJ. The agonists were dissolved in DMSO and diluted with PBS, and administered at a dose of 300 µg/kg, as recommended by the Manufacturer. The agonists were given as a single s.c. injection per day, starting at the day of the operation. In the *in vitro* cell line studies, drug concentrations of 0.1, 1, 5, and 10 µM were used. In the *ex vivo* explant studies, the drug concentrations were 300 µg/kg *in vivo* and 10 µM *in vitro*. For *in vitro* studies, the compounds were dissolved in DMSO, and a 25 mM stock solution was prepared. The final DMSO concentration in the *in vitro* studies was always lower than 0.05%. Selectivities of the different somatostatin analogs are summarized in Table 5.

Table 5 *Selectivity of somatostatin analogs in Ki (nM) as measured in CHO-K1 cells.*

Analog	sst_1	sst_2	sst_3	sst_4	sst_5	Reference
Nonselective natural agonist						
SST-14	0.4-1.1	0.04-1.3	0.7-1.6	0.53-1.7	0.9-2.3	Patel and Srikant 1994 Rohrer et al. 1998
sst_2/ sst_3/ sst_5-selective peptide agonist						
Octreotide	230->1000	0.27-2.1	4.4-45	>1000-2191	5.6-137	Patel and Srikant 1994 Yang et al. 1998
sst_1/ sst_4-selective peptide agonist						
CH275	3.2	>1000	>1000	4.3	>1000	Patel 1997
Non-peptide agonists						
L-797,591	1.4	1875	2240	170	3600	Rohrer et al.1998
L-779,976	2760	0.05	729	310	4260	Rohrer et al.1998
L-796-778	1255	>10.000	24	8650	1200	Rohrer et al.1998
L-803,087	199	4720	1280	0.7	3880	Rohrer et al.1998
L-817,818	3.3	52	64	82	0.4	Rohrer et al.1998

Somatostatin plasma levels (II)

The somatostatin radioimmunoassay (RIA) kit was obtained from Eurodiagnostica (Malmö, Sweden). Trifluoroacetic acid (TFA; 1% vol/vol, for CH275) or hydrochloric acid (HCL, 0.1 M, for SST-14) was added to each sample and the solution was passed over a disposable SepakC18 (Waters, Milford, MA) column. Trapped peptides were eluted with 100% methanol. The two peptides were also separated by high-pressure liquid chromatography by using a DeltaPak HPI C 18 (Waters) column (2 mm x 15 cm, 5 mm particle size). The fractions were collected, evaporated, and redissolved in RIA assay buffer and RIA was performed.

Sirolimus and imatinib (IV)

Sirolimus (Rapamune oral solution, Wyeth Europa, Berkshire, UK) and imatinib (Glivec capsules, Novartis, Basel, CH) were administered orally once a day via a curved gavage needle. Sirolimus was stored in the dark at +4°C and used as prescribed; imatinib was dissolved in vehicle (PBS) at a concentration of 10mg/mL, stored at +4°C, and used within 3 days. The rats were treated with (1) sirolimus 1.0 mg/kg/day, (2) imatinib 10.0 mg/kg/day, (3) the combination of these, or (4) vehicle. The dosages were based on previously performed dose-response analyses (Vamvakopoulos et al. 2006), and sub-maximal doses were chosen for this study (Fig. 7). Treatment was initiated three days before the operation and terminated on day 14 post injury. As in the clinic, the sirolimus-treatment was started with a loading dose of 3.0 mg/kg/day.

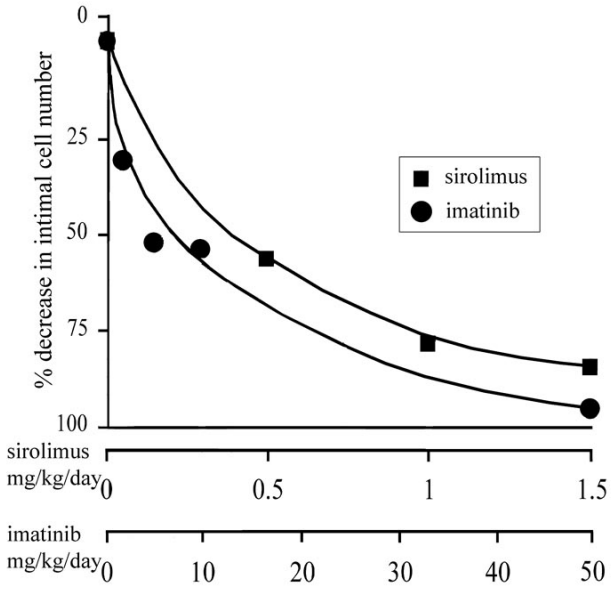

Figure 7 *Dose-response curves of sirolimus and imatinib monotreatment, 14 days post injury. Submaximal doses were chosen for this study. Modified from Vamvakopoulos et al. J Vasc Res 2006;43:184-192, with permission of S. Karger AG, Basel, CH.*

3. Cell culture studies

Cell lines (II and unpublished results)

Rat aortic SMCs were isolated from 4-day old Wistar rat aortas using a method modified from that of Thyberg and coworkers (Thyberg et al. 1983). The aortas were opened longitudinally and the endothelial layer was gently scraped off. The adventitia and media were separated, and the medial layer was digested with 0.1% collagenase and DNAse in PBS for 30 min at +37ºC. The cells were centrifuged, suspended in culture medium and allowed to attach to plastic flasks. Primary cells were used at passage 11-13 for the experiments. For identification, the cells were grown on glass slides and stained for smooth muscle α-actin (SMA).

The A10 rat smooth muscle cell line from embryonic thoracic aorta and the MCF-7 human cell line derived from mammary carcinoma were obtained from the American Type Culture Collection (Manassas, VA).

Human primary skin fibroblasts were isolated from skin tissue of cadaveric human donors. The tissue was cut into pieces with scissors and placed in plastic flasks with culture medium, allowing fibroblasts to grow out of the tissue pieces. For the experiments, primary fibroblasts were used at the 11-13th passage. For identification, the cells were grown on glass slides and stained for vimentin, SMA and von Willebrandt factor (vWF). All cells expressed vimentin, while none of the cells stained positive for SMA or vWF.

The culture medium used in experiments with primary SMCs, A10 cells, and primary human fibroblasts consisted of Dulbecco's Modified Eagle's medium (DMEM, Gibco, Paisley, Scotland) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), 100 IU/ml penicillin and 100 µg/l streptomycin, and 2 mmol/ml L-glutamine. For MCF-7 cells, a 1:1 mixture of DMEM and Nutrient Mixture F-12 (DMEM/F-12, Gibco) with 5% of FBS was used. All *in vitro* studies were done in triplicate and repeated three times.

Quantitation of cell proliferation *in vitro* (II and unpublished results)

In vitro DNA synthesis was measured by tritiated thymidine (^{3}H-TdR, Amersham Pharmacia, Amersham, UK) incorporation in response to 5% FBS. Cells were seeded on 96-well plates (Nunc, Roskilde, Denmark) at a concentration of 5000 cells/well and allowed to adhere to the wells in the culture medium supplemented with 5% FBS. The next day, the medium was changed to a medium containing 0.5% FBS, and the cells were starved for 24-72 hours. Thereafter, the starvation medium was replaced with culture medium containing 5% FBS as a stimulant, the ^{3}H-TdR labeling solution, and the serially-diluted drugs. The cells were allowed to proliferate for 24-72 hours before the content of each cell was harvested using trichloroacetic acid (TCA) and natriumhydroxide (NaOH), and mixed with OptiPhase Hisafe (LKB-Wallac, Turku, Finland). For the cell toxicity assay, the drugs were removed after 48 hours, and the cells were allowed to proliferate in culture medium containing 5% FBS without drugs for another 48 hours, before the

experiment was terminated. Finally, the amount of radioactive-labelled thymidine incorporated to the DNA was measured with a Rackbeta liquid scintillation counter (LKB-Wallac).

Quantitation of cell migration *in vitro* (II and unpublished results)

Migration was quantitated using Transwell culture chambers (Costar, Cambridge, MA). The Transwell system consists of an upper and a lower chamber separated by a polycarbonate filter with 8-µm pores. The chambers were first coated with collagen (20 µg/ml; Rat Tail Collagen, Type 1; Upstate Biotechnology, Lake Placid, NY), at +4ºC for 24 hours. The cells were seeded in the upper chamber at a concentration of 50,000 cells/chamber, with 60 ng/ml of the chemoattractant PDGF-BB (Upstate Biotechnology) added to the lower chamber, and culture medium without FBS added to both chambers. In the upper chamber the cells were allowed to adhere to the filter for 60 minutes, and the drugs were then added to the upper chamber. After 24 hours at +37ºC, the cells that had migrated to the lower side of the filter were fixed in methanol (+4ºC), stained with hematoxylin, and counted microscopically using 400 x magnification (Fig. 8).

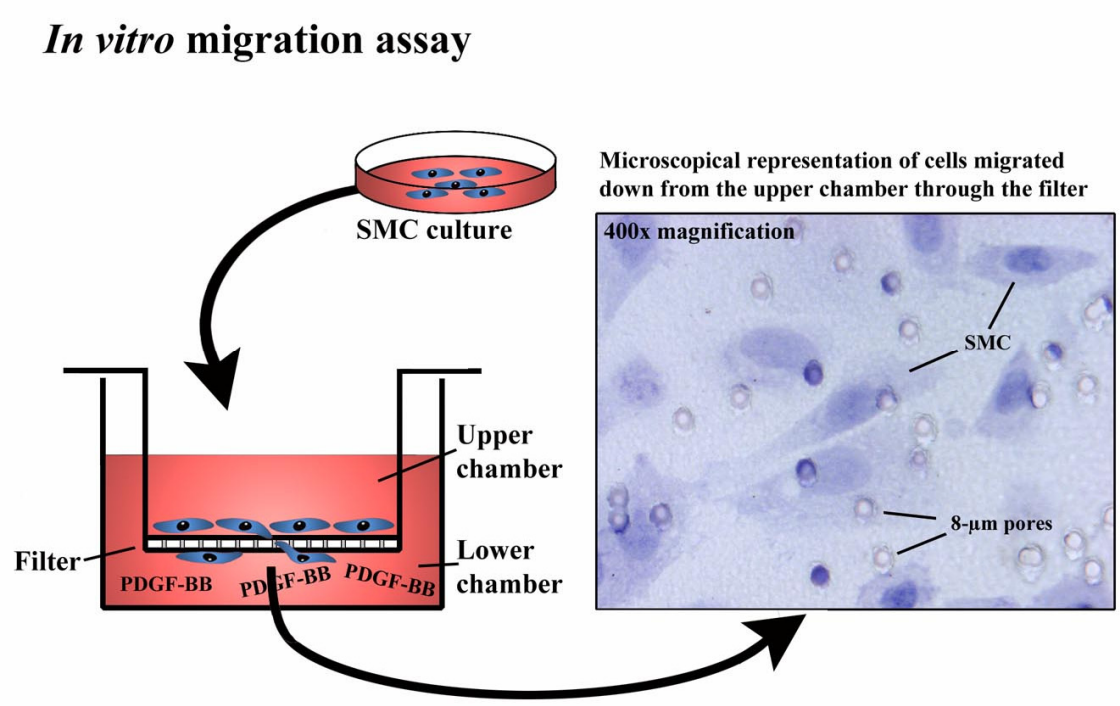

Figure 8 *Principle of the Transwell migration assay using cultured SMCs. The cells were stained with hematoxylin to visualize cell nuclei.*

4. Tissue culture studies

Preparation of aortic explants (I-IV)

As a significant part of neointimal cells have been suggested to originate from circulating precursors (Sata et al. 2002), isolated medial SMC cultures might not represent the real neointimal cell population, and experiments with these cells might give biased results. Therefore, a whole vessel explant assay was set up not to exclude any cell type when studying cell migration and proliferation (Fig. 9). The thoracic aortas or carotid arteries of injured and control animals were dissected under sterile conditions using a method modified of that described by Grunwald and coworkers (Grunwald et al. 1984). The vessels, including all vascular layers, were dissected longitudinally and explants measuring 1 x 1 mm were prepared with a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The explants were then placed individually into the wells of flat-bottomed 96-well plates (Nunc). To keep the explants damp, 20 µl basal medium consisting of DMEM (Gibco), 10% FBS (Gibco), 100 IU/ml penicillin and 100 µg/l streptomycin and 2 mmol/ml L-glutamine, was added. The plates were placed in a +37°C incubator with an atmosphere of 95% air and 5% CO_2 for 2 h to allow adherence to the tissue culture plastic, whereafter 200 µl of culture medium with or without the drugs studied was added to each well.

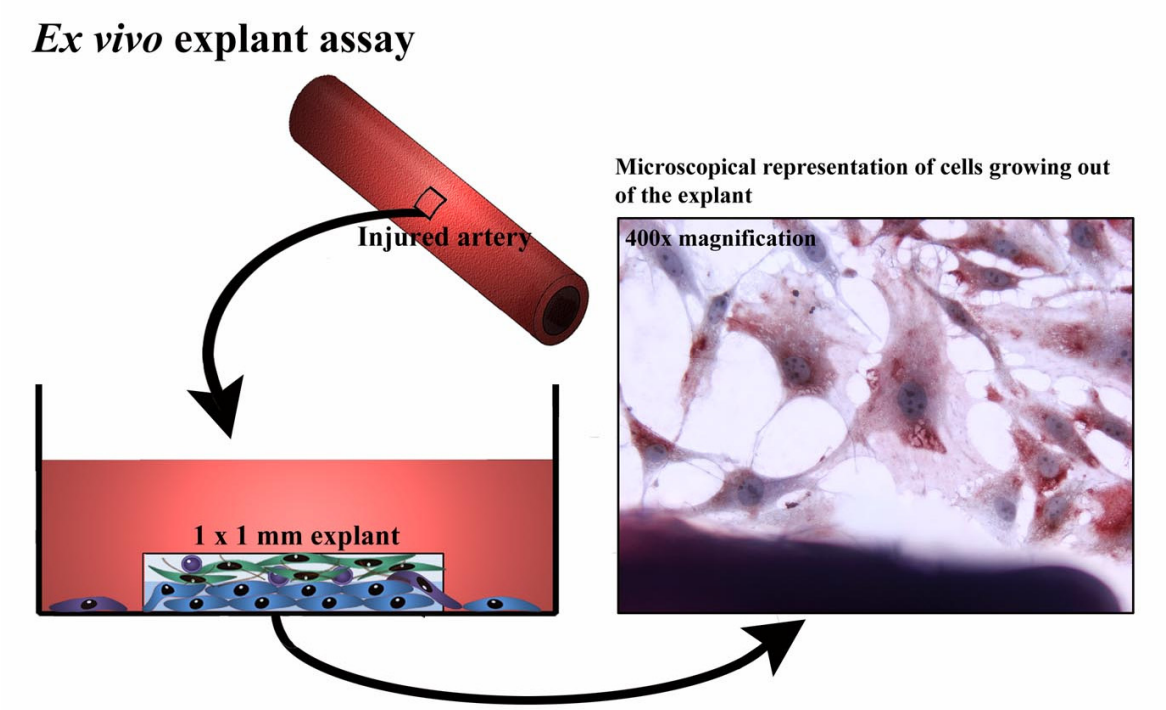

Figure 9 *Principle of the ex vivo explant assay. Whole vessel explants were used in the study not to exclude any cell type from the proliferation and migration measurements.The cells growing out of the explant have been double stained for SMA (red) and vWF (black), hematoxylin counterstain.*

Measurement of explant outgrowth, migration, and proliferation (I-IV)

Each well was observed daily and counted as positive for sprouting if cells had grown out of the explants. Migration was measured with a calibrated graticule (Olympus, Tokyo, Japan) as the distance that the leading edge of sprouting cells in each well had traveled from the explant. To measure cell proliferation, the explants were pulsed with ^{3}H-TdR on day 0. On day 2 or 4, the outgrowing cells were detached from the wells with trypsin-EDTA (Gibco), and the explants were digested with pepsin (Merck, Darmstadt, Germany) for 50 min at +37°C. The content of each well was subsequently harvested with a Dynatech Harvester (Dynatech Labs, Sussex, UK) and mixed with OptiScint Hisafe (LKB-Wallac) before measuring radioactivity with a Rackbeta liquid scintillation counter.

Limiting dilution assay (I)

To evaluate the influx of precursors to the vessel wall after injury, explants taken at 15 minutes, 2, 4, 14, and 21 days post injury were cultured under limiting dilution conditions. Samples measuring 1x1 mm, 0.5x0.5 mm, 0.3x0.3 mm and 0.1x0.1 mm were obtained using the McIlwain Tissue Chopper and cultured as described above, and the outgrowth of cells was measured after 24 hours of culture. Estimates of precursor frequencies in the vessel wall at different time points post injury were calculated according to the Poisson distribution equation as the slope of a line relating the number of cells per microwell (plotted on a linear x-axis) and the percentage of wells with no outgrowth (plotted on a logarithmic y-axis) (Dozmorov et al. 2000).

5. Histological evaluation

Morphometric analysis (I-IV)

For evaluation of morphological changes, aortic and carotid midsection specimens were fixed in 3% paraformaldehyde, processed for paraffin embedding, and stained with Mayer's hematoxylin and eosin, Masson's connective tissue, and silver elastic stainings. The number of cells in the intima, media, and adventitia, and the number of microvessels in the adventitia, were calculated from paraffin cross sections at 400x magnification. The areas of the intima and media, and the lumen area and perimeter length were quantitated using an Olympus video microscope and the Macintosh NIH-Image Ver.1.62 or Windows Image-Pro Plus Ver.4.1.0.0 softwares.

Quantitation of cell proliferation *in vivo* (II and unpublished results)

Numbers of proliferating cells *in vivo* were quantitated with the 5-bromo-2'-deoxyuridine (BrdU) method. Proliferating cells were labelled with the BrdU solution (3mg/mL, Pharmacy of Helsinki University Central Hospital, Helsinki, Finland) intravenously 3 h before sacrifice. Incorporation of the labelling reagent was visualized by immunohistochemistry (Table 6) using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), according to the Manufacturer's instructions. Samples were counterstained with Mayer's hematoxylin and eosin, and the number of positive cells was counted separately from the intima, media, and adventitia using 400x magnification.

Immunohistochemistry (I, IV)

Immunohistochemistry was used to localize different vascular cell markers, and proteins coding for the genes quantitated with PCR. A summary of the antibodies and dilutions used is presented in Table 6. Paraffin-embedded vessel specimens were deparaffinized before the stainings. The stainings were performed using a commercial avidin-peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories). The slides were sequentially incubated with 0.1% Triton X-100 (5 min, room temperature, RT; Sigma), 1.5% goat, horse, or rabbit serum (Vector Laboratories; 30 min, RT), primary antibody (60 min, RT), biotinylated secondary antibody (30 min, RT), avidin-peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories; 30 min, RT), 0.1% hydrogen peroxide, and 3-amino-9-ethylcarbazole (AEC; Sigma), or Nickel-3,3'-diaminobenzidine (Ni-DAB; Zymed Laboratories, Inc., San Francisco, CA). Finally, the specimens were counterstained with hematoxylin, and coverslips were mounted (Aquamount, BDH Ltd., Poole, UK).

Controls for antibody staining included replacing the primary antibody with irrelevant antibody (M0744, DAKO, Glostrup, Denmark), and omitting the primary antibody. The immunostaining was graded separately from the intima, media, and adventitia as follows: 0 = no visible staining; 1 = some cells with weak staining; 2 = moderate staining with multifocal expression; 3 = intense staining throughout the vessel compartment.

Immunohistochemistry was also used for identification of cells growing out of aortic explants. Tissue culture treated glass slides (Becton Dickinson and Co., Franklin Lakes, NJ) were coated with collagen (Rat Tail Collagen, Type 1, Upstate Biotechnology) at a concentration of 20 µg/ml at +4°C for 24 h. Aortic explants were prepared as described, and 3-5 tissue pieces were placed per well to obtain enough material for the stainings. Slides were incubated with 50 µl of culture medium in each well in +37˚C for 4 h to allow the explants to adhere to the slides. Thereafter, 250 µl of culture medium was added and the explants were cultured for 7 days. On day 7, the explants and cells were fixed with 3% paraformaldehyde solution for 15 min, whereafter the slides were kept damp with sterile PBS at +4˚C until stained. The stainings were performed as described above, and the number of positive cells was counted microscopically using 400x magnification.

Table 6 *Summary of antibodies used for immunohistochemistry.*

Antibody	Clone	Marker	Dilution	Manufacturer
Mouse monoclonal antibodies				
BrdU	Bu20a	Proliferating cells	1:20	DAKO, Glostrup, Denmark
ED1	22451D	Monocytes/macrophages	1:200	BDPharmingen, San Diego, CA
Flk-1	sc-6251	Hematopoietic/endothelial precursors	1:100	Santa Cruz Biotechnology, Inc., Santa Cruz, CA,
LCA	M0701	Leukocytes	1:50	DAKO
SM22α	10H12	SMC differentiation	1:100	Novocastra, Newcastle upon Tyne, UK
SMA	A2547	SMCs	1:200	Sigma, St. Louis, MO
Vimentin	M0725	Cells of mesenchymal origin	1:100	DAKO
Rat monoclonal antibody				
CD34	sc-52478	Hematopoietic precursors	1:100	Santa Cruz
MECA-79	553863	High endothelial venules	1:50	BDPharmingen
HECA-452	550407	High endothelial venules	1:50	BDPharmingen
Rabbit polyclonal antibodies				
BCL2	RDI-BCL2abr	Cell survival	1:100	Fitzgerald, Concord, MA
vWF	A0082	Endothelial cells	1:100/1:200	DAKO
Chicken polyclonal antibody				
CSRP2	ab14011	SMC differentiation	1:200	AbCam, Cambridge, UK
Goat polyclonal antibody				
CD11b	sc-6614	Monocytes, macrophages, activated lymphocytes	1:100	Santa Cruz

6. Quantitative real-time PCR (QRT-PCR)

Total RNA was extracted with RNeasy Mini spin columns (Qiagen GMbH, Hilden, Germany), and RNA quality was analyzed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). RNA amplification was performed using an established technique (Wang

et al. 2000). Amplified anti-sense RNA was reverse transcribed to cDNA using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA).

QRT-PCR analysis was carried out using RotorGene 3000 (Corbett Life Science, Sydney, Australia) apparatus and SYBR Green chemistry. The QRT-PCR samples consisting of cDNA, both primers, and PCR Master Mix (Applied Biosystems, Foster City, CA), were cycled 40 times at the following conditions: denaturation (+95°C, 10 min), annealing (+60°C, 30 sec), extension (+72°C, 30 sec), and denaturation (+95°C, 15 sec). The final extension was carried out at +72°C for 10 min. Gene expression levels were calculated using RotorGene software (Corbett Life Science) by a comparative quantitation algorithm. Primer sequences and accession numbers have been given in the original publication (IV).

7. Complete blood counts (IV)

Whole blood samples were collected from rats at sacrifice in EDTA tubes for a complete blood count analysis, including white blood cell count, white blood cell differential, red blood cell count, hemoglobin, hematocrit, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Samples from untreated and unoperated rats were collected to obtain normal values. All samples were analyzed at the Central Laboratory of the Department of Clinical Veterinary Sciences, University of Helsinki, Finland, with a Cell-Dyn 3700 System hematology analyzer (Abbott Laboratories, Santa Clara, CA).

8. Statistical methods

The data are given as mean±SEM. Limiting dilution analysis was performed with the L-Calc Software (Stem Cell Technologies, Vancouver, Canada). Statistical analysis of parametric comparisons was performed with the Students t test or one-way ANOVA with Dunnett's or Bonferroni correction. In the remaining cases the Mann-Whitney test or Kruskal-Wallis test with Dunn correction were applied. The software used for the analyses was Statview 5.0.1 (SAS Institute Inc., Cary, NC). $P < 0.05$ was considered statistically significant.

RESULTS

1. The *in vivo* and *ex vivo* response to vascular injury

The *in vivo* response to denudation injury (I)

The catheter-induced aortic denudation injury resulted in a complete loss of intimal endothelial cells. On day 4, the first neointimal cells started to appear, and between days 7 and 14, the intimal cell number increased markedly, whereafter a plateau was reached. The endothelial injury did not induce changes in the medial cell number at any time point. However, between days 4 and 14, the adventitial cell number increased up to 4-fold compared to control and returned to control level by day 28 post injury (Fig. 10). Also, the adventitial microvessel number increased 4-fold between days 7 and 21, whereafter their number returned back to the pre-denudation level.

For further evaluation of the response to denudation injury, aortic samples were stained for the endothelial cell-specific antigen vWF, the SMC-specific SMA, the cytoskeletal protein vimentin, and the progenitor cell markers Flk-1 and CD34. In un-injured vessels, vWF was predominantly expressed in the intima. Re-endothelialization of the neointima started between days 4 and 14 post injury, and was nearly completed by 90 days post injury.

Medial cells expressed SMA both in the resting state and post injury. The first SMA-positive neointimal cells were detected at 7 days after injury. On day 14, the whole neointima stained faintly positive. Thereafter, the staining intensity increased, and from 21 to 28 days post injury, both the neointima and media showed strong immunoreactivity for SMA.

Vimentin was expressed in all layers of the resting vascular wall. Early after injury, the expression was mainly localized to the adventitia. However, the intensity of adventitial staining declined by day 21, and thereafter, vimentin was predominantly expressed in the neointima.

Flk-1 was expressed in the adventitia and occasionally in intimal cells of un-injured vessels. After injury, the first neointimal cells stained positive for Flk-1, and at later time-points staining was seen in all vascular layers. Our attempts to show CD34 protein in any of the samples failed.

The *ex vivo* response to denudation injury (I, II)

An *ex vivo* aortic explant model was established to evaluate the migration, proliferation, and maturation of neointimal cells in an *in vitro* setting without excluding neointimal precursor cells.

The percentage of wells with sprouts, the migration distance, and the frequency of progenitor cells increased significantly in samples obtained 2-14 days post denudation, peaking on day 4 post injury (13-fold, 3.5-fold, and 8.5-fold changes compared to control, respectively). DNA synthesis in explants, as measured by ^{3}H-TdR incorporation, peaked on day 7 after denudation injury (6771±452 CPM vs. 3700±466 CPM, $p<0.0001$ compared to control). Thereafter, the explant activity declined and returned to the control level by day 21 after injury (Fig. 10).

The cells growing out of vascular explants were identified by immunohistochemistry. The majority of the outgrowing cells expressed SMA and a significant part also expressed vWF, vimentin, or the progenitor cell markers Flk-1 and CD34. Only 0.05±0.015% of the cells were positive for the mononuclear phagocyte antigen ED1 and 2.6±2.5% for the leukocyte antigen LCA. Double immunohistochemistry showed that many of the cells co-expressed SMA, vWF, vimentin, or LCA.

The cellular responses in carotid and aortic explants were also compared and they were shown to be similar (II). As it was difficult to obtain enough tissue material from carotid arteries, only aortic explants were used in the subsequent studies (I, III, IV).

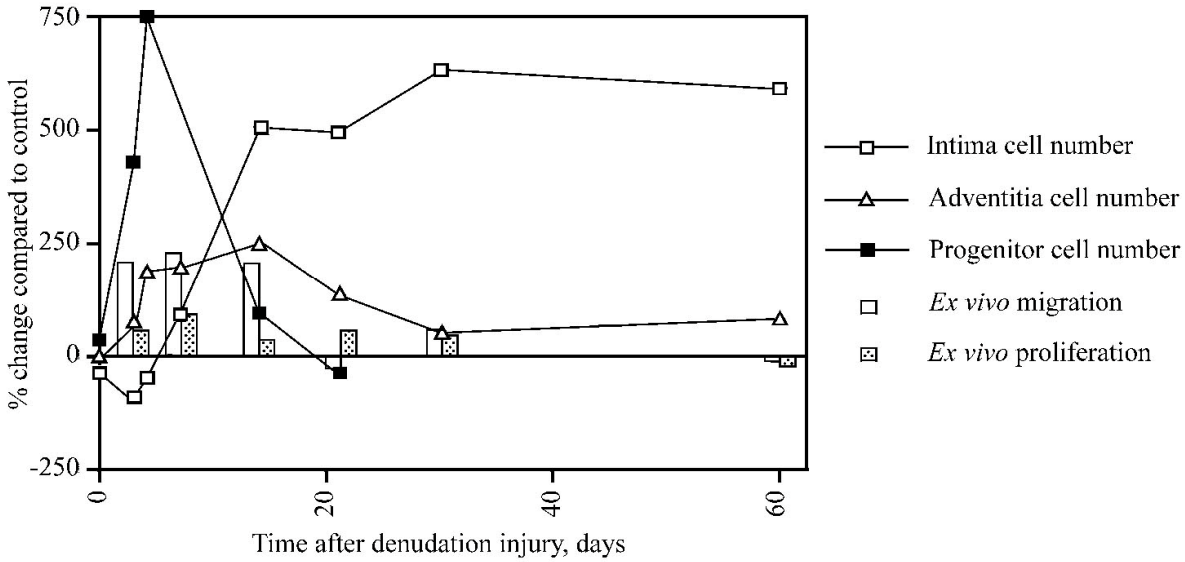

Figure 10 *Kinetics of intima and adventitia cell numbers in vivo after denudation injury compared to progenitor cell numbers and cell migration/proliferation in ex vivo explants.*

2. The vasculoprotective effects of somatostatin and its analogs

Effects of somatostatin and its analogs on neointimal hyperplasia (II, III)

The nonselective SST-14, administered as daily s.c. injections, significantly decreased the intimal cell number ($p=0.001$ compared to control), intimal area ($p=0.03$), and reduced the intima-media ratio ($p=0.014$). CH275, selective to sst_1 and sst_4, offered superior

vasculoprotection, and dose-dependently decreased the intimal cell number (p=0.0001 compared to control), intimal area (p= 0.0002), intima-media ratio (p=0.0009), as well as the adventitial cell number (p=0.006). CH275 also decreased the number of replicating cells in the intima, as measured by BrdU-incorporation (p=0.004). Octreotide, selective to sst_2, sst_3, and sst_5, decreased only the intimal cell number (p=0.01).

SST-14 and CH275 had no effect on neointimal hyperplasia when administered as continuous infusions through osmotic minipumps.

To investigate if discontinuation of the medication would cause a rebound effect, rats received SST-14, CH275, and octreotide for 14 days post injury, whereafter the medication was replaced with vehicle for another 14 days. In this setting, no rebound effect on intimal hyperplasia was observed with CH275 and octreotide, while SST-14 lost its efficacy.

As CH275, selective to sst_1 and sst_4, proved to be the most effective compound in inhibiting neointimal hyperplasia, we continued to investigate the individual vasculoprotective properties of sst_1 and sst_4 with subtype specific agonists. Daily s.c. injections of the sst_4-selective agonist remarkably increased lumen size ($p<0.01$), and reduced the intima-media ratio ($p<0.05$), the intimal cell number ($p<0.05$), and intimal area ($p<0.05$). The sst_1-selective agonist increased lumen size ($p<0.05$ compared to control), while the other sst-selective agonists had no effects on the parameters measured. None of the subtype-selective agonists affected cell proliferation *in vivo* (Tigerstedt NM et al. unpublished results).

Effects of somatostatin and its analogs on *ex vivo* explants (II, III)

The explant model was used to evaluate the effect of the drugs *ex vivo*. The explants were obtained two days post-injury and outgrowth, migration, and proliferation were evaluated after 48 h of culture. The animals were given SST-14, CH275, or octreotide *in vivo* at a dose of 500 µg/kg, and the treatment was continued *in vitro* at a dose of 10 µM, or with vehicle. CH275 and octreotide inhibited cell outgrowth and migration, but not cell replication in the post injury tissue fragments. SST-14 did not have significant effects on any of the measured parameters.

In the explant experiment with the subtype-selective sst agonists, the drugs were administered *in vivo* only (III). Only the sst_4-selective ligand inhibited explant outgrowth by 42% and migration by 60% ($p<0.01$ and $p<0.05$, respectively). The sst_5-selective agonist had a small inhibitory effect on explant outgrowth ($p<0.05$), but none of the other agonists had any effect on the parameters measured.

In vitro cellular responses to drug treatment (II, III, and unpublished results)

The effects of the somatostatin analogs were also tested using the ^{3}H-TdR replication and Transwell migration assays with primary rat SMCs and the A10 rat SMC line and, for comparison, human skin fibroblasts and the MCF-7 human breast cancer cell line. SST-14,

CH275, and octreotide did not affect the proliferation of the cell lines used. Only the sst_1-selective compound showed a significant antiproliferative effect on all the cell lines at the 10 µM concentration (Fig. 11A, Tigerstedt NM et al. unpublished results).

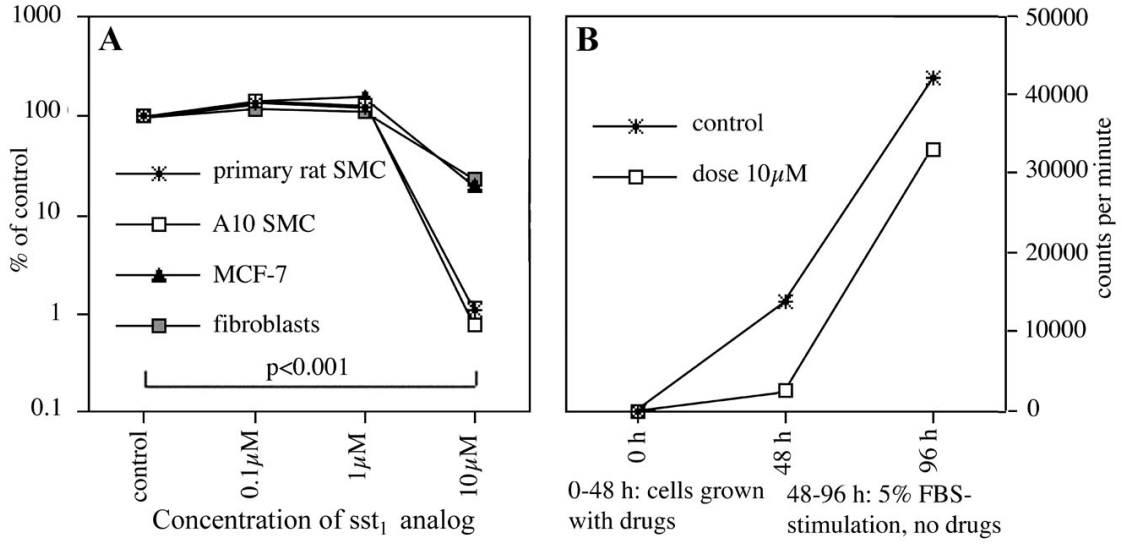

Figure 11 *Effect of the sst_1-selective analog on the proliferation of SMCs, MCF-7 cells, and fibroblasts.*

However, the 10 µM dose was not toxic, as the inhibitory effect of the sst_1-selective compound was reversible, and there was no difference in the viability of the drug-treated cells compared to the non-treated cells after 48 h of culture *in vitro* (Fig. 11B, Tigerstedt NM et al. unpublished results).

In the Transwell chamber assay, none of the drugs had any effect on the PDGF-BB (60 ng/ml) induced migration.

Effects of somatostatin and its analogs on rat weight and drug levels (II, III)

SST-14 and the somatostatin analogs were well-tolerated, and there were no weight differences between vehicle and drug-treated rats.

The serum levels of SST-14 and CH275 were evaluated as the drugs inhibited neointimal hyperplasia only when administered as daily injections, but not when administered through osmotic minipumps. SST-14 concentration peaked at 360 pmol/L, and CH275 concentration at 280 pmol/L after 200 µg/kg s.c. injections. Estimated *in vivo* half-lives were 20 min for SST-14 and 90 min for CH275. Using minipumps, serum levels of approximately 110 pmol/L were obtained at about 10 h after installation of the pump, and they remained on this level throughout the 7-day lifetime of the pump. With the highest drug dose, 500 µg/kg, high (up to 1200 pmol/L) but short lasting peaks were observed.

3. The vasculoprotective effects of sirolimus and imatinib

Long-term effects of sirolimus and imatinib on neointimal hyperplasia (IV)

When given individually, only sirolimus showed moderate vasculoprotective effects throughout the observation period while imatinib lost its effect after 14 days post-injury. However, early combination treatment with sirolimus and imatinib, discontinued at day 14 post-injury, showed a sustained therapeutic synergy that was statistically significant even at 90 days with 64% suppression in intimal nuclei number ($p<0.0001$ compared to control), 82% suppression in intimal area ($p=0.0048$), and 13% increase in perimeter length ($p=0.0103$) (Table 7). No changes were seen in medial and adventitial nuclei numbers or areas.

Table 7 *Effects of drug treatment on intimal cell area, cell number, and perimeter length after denudation injury.*

	Control	Imatinib	Sirolimus	Combi
Intimal area (square pixels x10^3)				
Day 14	74.4±11.9	46.4±6.0	31.1±5.7*	18.0±1.4†
Day 40	74.0±14.0	59.8±12.5	30.0±4.2*	12.8±3.5†
Day 90	85.2±15.4	61.7±13.7	41.4±6.8*	15.4±9.4†
Intimal cell number				
Day 14	872.2±38.1	585.6±41.0‡	371±43.9‡	142.8±8.2‡
Day 40	803.8±77.2	653.3±80.6	366.1±54.4‡	159.6±20.2‡
Day 90	844.6±94.1	639.6±61.3	509.8±56.8†	301.8±64.9‡
Perimeter length (pixels x10^2)				
Day 14	43.3±0.7	45.7±1.4	49.0±1.2†	49.2±0.7†
Day 40	47.1±0.6	48.3±1.6	50.7±1.4	51.3±0.9†
Day 90	47.6±1.1	49.4±1.9	51.0±0.9	53.8±1.2*

The data are expressed as mean±SEM (n=5-7). *p<0.5, †<0.01, ‡p<0.001, compared to control. Combi = combination therapy

Effects of sirolimus and imatinib on *ex vivo* explants (IV)

To study the effect of sirolimus, imatinib, the combination of these, or vehicle on *ex vivo* explant outgrowth, migration, and proliferation, the treatment was initiated 3 days before the operation and discontinued at sacrifice. Both imatinib and sirolimus inhibited explant outgrowth by 71% and 85%, respectively ($p<0.0001$ compared to control), and migration by 76% and 84%, respectively ($p=0.0005$ and $p=0.0002$). However, the effect of the combination treatment was superior, and it inhibited explant outgrowth by 93% and migration by 96% ($p<0.0001$). All treatments also reduced explant proliferation

($p<0.0001$); here sirolimus alone was the most effective treatment causing a 97% reduction in proliferation.

Effects of sirolimus and imatinib on vascular gene expression (IV)

Gene and protein expression in the vascular wall were assessed to evaluate possible mechanisms behind the effects of the different treatments.

The combination treatment with sirolimus and imatinib inhibited the early downregulation of the SMC markers SM22α and CSRP2, which are associated with the differentiated SMC phenotype. By immunohistochemistry, the SMC marker proteins were localized to the media on day 4 post injury, and on day 40 to the media and neointima.

Both the sirolimus and the combination treatments were associated with an increased expression of the anti-apoptotic BCL-2 mRNA on day 4 post injury compared to the control level. This was reflected mainly by increased BCL-2 immunoreactivity in the media and adventitia. On day 40 post injury, a downregulation of the BCL-2 mRNA was observed in all treatment groups. This was reflected by a lower BCL-2 staining intensity in the media.

Following denudation injury and a complete removal of the intimal endothelial layer, the vWF mRNA was highly downregulated on days 2-5 in control rats, and returned to the pre-injury level on days 5-7 when the reendothelialization of the intima begun. Sirolimus treatment upregulated the early vWF mRNA expression, while imatinib treatment repressed it, and the expression remained at the control level in the combination therapy group. At day 4 post injury, vWF protein expression was localized to the first cells re-appearing to the intima, as well as to the adventitial microvessels. While the vWF mRNA expression remained at the control level in the imatinib group on day 40 post injury, sirolimus and combination treatment both downregulated the vWF expression. This was observed as a delayed reendothelialization of the neointima by 62% ($p=0.0469$, compared to control) in the sirolimus treatment group, and by 54% ($p=0.0423$, compared to control) in the combination treatment group.

Effects of sirolimus and imatinib on rat weight and complete blood counts (IV)

The imatinib, sirolimus, and the combination therapy were well-tolerated, and there were no weight differences between vehicle and drug-treated rats. Complete blood counts were determined at days 4, 14, 40, and 90 post injury to gain mechanistic insights and to monitor the well-being and post-operative recovery of the animals. Blood hemoglobin levels decreased at day 4 after the operation in all but the combi group. By day 40 post-injury, the hemoglobin levels in all treatment groups had returned to pre-operation and pre-treatment level.

Blood leukocyte counts increased in the control group peaking at day 14 post injury. The same trend was seen in all other groups, except in the combi group, where the

leukocyte count remained at the pre-injury level. The elevation in leukocyte concentration was mainly due to an increased number of neutrophils and lymphocytes. There were no significant differences between the treatment groups at the later time points.

There was a tendency towards a rise in thrombocyte counts post injury at 4 days in all groups, except in rats treated with the combination of sirolimus and imatinib. At days 14, 40, and 90 post-injury, there were again no significant differences between the treatment groups.

DISCUSSION

Neointima formation after vascular injury is a complex interplay of different cell types, growth factors, cytokines, and signaling cascades. Despite extensive efforts to reveal the exact mechanisms behind this process and to develop therapeutic interventions, much is still unknown, and the treatment options are scarce.

1. Experimental models of vascular injury

In vivo models of intimal hyperplasia

Animal models are essential in restenosis research. Different stages of neointimal hyperplasia are impossible to study in humans, as the study material usually consists of post-mortem findings showing only severe disease. Furthermore, animal models are essential when screening for potential new drug candidates. The rat model of intimal hyperplasia after denudation injury used in this Thesis (I-IV) has several benefits. The model is well characterized, and it has been used extensively for several decades (Clowes et al. 1986, Myllärniemi et al. 1997). It is feasible, economical, reproducible, and technically relatively easy and fast to perform, which makes it less susceptible to methodological problems and biases. The rat vessel develops substantial levels of neointima, which is quite essential when studying intimal hyperplasia, but something often not seen in the standard mouse vascular injury models (Iafrati et al. 1997, Xu et al. 2004).

However, the rat model has some significant shortcomings. First, the vessel structure in rat differs from that in humans. Human vessels have a more prominent intima, while the rat vessel intima consists of a single layer of endothelial cells only (Sims 1989, Du Toit et al. 2001). Second, rats do not develop atherosclerosis. In clinical conditions, vascular injury includes a response to a long-term stress caused by factors such as low-grade inflammation, diabetes, dyslipidemia, and toxins. These are all absent in laboratory rats. Third, in the rat denudation injury model, the vascular injury is performed on a previously healthy artery, while the artery undergoing surgical interventions in the clinical setting is already diseased. Fourth and last, the therapeutic targets – signaling pathways, growth factors and receptors – might not be identical in rat and man. Nevertheless, if these limitations are acknowledged, the rat denudation model can provide important information about vasculoproliferative disorders and potential treatments for the prevention of such diseases.

In vitro and *ex vivo* models of intimal hyperplasia

Targeting the proliferation and migration of medial SMCs has been studied extensively. Traditionally, this has been performed using SMC cell lines or primary SMCs of medial

origin, and these cells have been observed in *in vitro* experimental set-ups, such as the Transwell chamber migration assay or ^{3}H-TdR replication experiments also used in this thesis study (II, Tigerstedt NM et al. unpublished results). Still, the efforts to target medial SMCs have not resulted in findings that could offer a solution for the treatment of vasculoproliferative disorders.

In 1984, Grunwald and Haudenschield observed that an *in vivo* intimal injury could stimulate cells to grow out of medial explants in *ex vivo* cultures (Grunwald and Haudenschild 1984). Later, it was also observed that hypertension increased proliferation in explants prepared in the same way (Grunwald et al. 1987). Whole vessel cultures were used in the 1960s and 1970s for various purposes (Wexler et al. 1967, Larrue et al. 1977), but later on the adventitia and intima were systematically dissected out (Kenagy et al. 1996, Sun et al. 2001, Kenagy et al. 2002), as neointimal cells were believed to originate from the media.

However, as a significant part of neointimal SMCs have recently been shown to derive from precursor cells of perivascular origin, the role of isolated medial SMC cultures or medial explants in restenosis research can be questioned. The limited liability of SMC cultures was also observed in this study. All of the somatostatin analogs tested, SST-14, octreotide, CH275, and the sst subtype-specific agonists, failed to prevent the migration of SMCs in Transwell cell chambers, and these results did not correlate with the potential of the compounds to prevent intimal hyperplasia *in vivo* (II, III, Tigerstedt NM et al., unpublished results). The same observation was made with the SMC replication experiments. Of the compounds mentioned above, only the sst_5-selective analog inhibited cell proliferation, but again, this finding could not be related to *in vivo* events (II, III, Tigerstedt NM et al., unpublished results).

To compensate for the limitations of the *in vitro* experiments, an *ex vivo* explant assay was set up. In this assay, the entire vessel wall was used for the culture to also include precursors migrated into the adventitia or neointima. A vascular injury was initiated *in vivo*, and later 1 x 1 mm punch explants were prepared for tissue culture, and monitored for cell outgrowth, migration, and proliferation (I). As evaluated by immunohistochemistry, the cells growing out of the explants showed characteristics of undifferentiated cells, as they co-expressed SMA, vWF, LCA, or vimentin. Furthermore, almost half of the cells expressed the progenitor cell marker Flk-1, and 14% were positive for the stem cell marker CD34 (I). Results from somatostatin studies using the *ex vivo* explant assay showed a much higher correlation with the *in vivo* findings, compared to traditional *in vitro* studies (II, III). Despite the possibility of some contamination with irrelevant cells types, the *ex vivo* explant assay offered a more versatile approach to neointimal cell studies than the *in vitro* SMC studies or medial explant cultures.

2. Pathogenesis of vascular disease

Vascular SMC migration and proliferation

The major cell type in neointimal lesions is the SMCs that migrate into the intima, proliferate, and produce extracellular matrix, thus forming a neointima. The role of cell proliferation in this process has evoked controversies. In animal models, only a small fraction of SMCs proliferate in response to vascular injury, and migration seems to be more important than proliferation (Clowes and Schwartz 1985, Du Toit et al. 2001). Furthermore, cell proliferation is low in specimens retrieved from human restenotic atherectomy tissue or in-stent restenosis sites (Moreno et al. 1999, O'Brien et al. 2000).

These observations are consistent with the results of this thesis study. In the *ex vivo* explant model, the migratory response to vascular injury far outweighed the proliferatory response (I). Also, the potential vasculoprotective somatostatin analogs that could inhibit intimal hyperplasia *in vivo*, showed antimigratory properties in the *ex vivo* model. At the same time, their antiproliferative capacity, as measured by *in vivo* BrdU incorporation, or *in vitro* / *ex vivo* ^{3}H-TdR incorporation, was scarce, or close to non-existent (II, III, Tigerstedt NM et al. unpublished results).

Stem cells in vascular disease

The recent finding that neointimal SMCs may stem from perivascular progenitor cells has evoked much interest and speculations on the origin of the progenitor cells, as well as of their relative contribution to arterial repair compared to SMCs derived from the vessel wall. The highly differing results might be explained by SMCs having several sources of origin (Tanaka et al. 2003). It has also been proposed that the role of perivascular progenitors might vary depending on the severity of the vascular injury (Campbell et al. 2001, Tanaka et al. 2003). With the experimental setup used in this thesis study, it was unfortunately not possible to address the question on whether neointimal cells derive from the bone marrow or other perivascular tissue. However, in general, there seems to exist a wide acceptance for the perivascular origin of at least a significant part of the neointimal SMCs (Campbell et al. 2001, Sata et al. 2002, Tanaka et al. 2003, Xu et al. 2004).

The time-window for precursor cell influx is not known, and both the intima and adventitia have been suggested as routes for precursor cell entry to the vessel wall (Campbell et al. 2001, Saiura et al. 2001). In a rat denudation injury model, the proliferative activity of cells as measured by BrdU-incorporation has been observed to shift from the adventitia to the neointima by 14 days post-injury (Frosen et al. 2001; I). Also, in mice carotid allografts, the first cells of perivascular origin have been observed in the adventitia at 7 days after transplantation, whereas they do not appear in the neointima or media until one week later (Matsumoto et al. 2003). Moreover, the number of adventitial microvessels has been shown to increase acutely after vascular injury (Pels et

al. 1999, Cheema et al. 2006), reflecting adventitial activity and remodeling. Precursor cells have also been suggested to store up in the adventitia in aortic roots (Hu et al. 2004).

In this study, the influx of precursor cells occurred early after endothelial removal, mostly on days 2-4 (I). In concert with previous findings, formation of the neointima was preceded by adventitial activation, an increase in adventitial microvessel number, as well as a displacement of vimentin and Flk-1 expression from the adventitia to the neointima (I). Thus, the results of this Thesis further emphasize the role of the adventitia in the injury response and indicate that the adventitia might serve as a reserve, or a point of entry to the vascular wall for SMC precursors (I).

3. Therapeutic strategies to overcome restenosis

3.1 Somatostatin and somatostatin analogs

Somatostatin exerts its inhibitory action on growth hormone secretion, cellular proliferation, as well as different growth factors and cytokines through five GPCRs. Due to its limited bioavailability, a short half-life of less than 3 minutes, and multiple functions in different organs, numerous somatostatin analogs with improved pharmacokinetics and receptor selectivity and affinity have been developed both for therapeutic and investigational use. In the late 1980s, the vasculoprotective properties of somatostatin were observed (Lundergan et al. 1989). However, promising preclinical results with sst_2/sst_3/sst_5 analogs in the prevention of intimal hyperplasia could not be repeated in clinical trials (Emmanuelsson et al. 1995, Eriksen et al. 1995, von Essen et al. 1997), reducing the enthusiasm for the development of somatostatin-based therapies for the prevention and treatment of vasculoproliferative disorders.

Somatostatin receptor expression in vascular tissue

The pattern of somatostatin receptor expression shows high variation between different tissues and cell types (Hofland and Lamberts 2001, Reubi et al. 2001), and the literature is very inconsistent when it comes to sst receptor subtype expression profiles, especially as far as vascular cells are concerned. This has partly been suggested to arise from differences in detection sensitivity between the methods used (Moller et al. 2003). There are also significant species-dependent differences between the sst expression profiles (Olias et al. 2004), as well as variation between individuals (Adams et al. 2005, Taniyama et al. 2005). Furthermore, somatostatin receptor expression can change during disease progression (Khare et al. 1999), as a result of ligand binding (Rocheville et al. 2000a), or cell replication (Adams et al. 2005). The interpretation of results on sst expression obtained from different experimental setups in different species is thus a challenging task.

Most investigations on sst expression in human endothelial cells have been made on HUVECs, and although the results differ highly, HUVECs have been shown to express all five ssts (Curtis et al. 2000, Florio et al. 2003, Jia et al. 2003, Adams et al. 2004, 2005). Also in human coronary artery endothelial cells, a cell type closer to our target cells, the reported sst expression ranges from the expression of sst_4 only (Badway et al. 2004) to the expression of sst_1, sst_2, and sst_5 (Yan et al. 2005). Endothelial cell heterogeneity most likely accounts for some of the observed differences in sst expression (Conway et al. 2004), as well as changes in receptor expression profiles that can occur during cell culture in late passages (Curtis et al. 2000). Human vascular SMCs have been shown to express sst_1 and sst_2 *in vitro* (Curtis et al. 2000). Finally, in human arteries *in vivo*, the predominant sst receptor subtypes in both normal and atherosclerotic vessels are sst_1 and sst_4 (Curtis et al. 2000).

In rat arteries, the expression of all ssts has been reported (Khare et al. 1999, Reynaert et al. 2007). However, most of the sst expression has been located to medial and neointimal SMCs, not to endothelial cells. Rat aortic SMCs *in vitro* have been shown to express mainly sst_4 (Torrecillas et al. 1999), although experiments with sst_2/sst_3/sst_5-selective agonists suggest that cultured SMCs can express at least sst_5, possibly also sst_2 and sst_3 (Grant et al. 1994, Lauder et al. 1997).

Taking into consideration the variety of sst receptor expression, it is fully possible that vascular cells are capable of expressing all sst subtypes, but that their expression varies at different stages of vascular disease. Still, in the rat model of vascular injury used in this thesis study (II, III), the predominant somatostatin receptor subtypes expressed post injury are sst_1, sst_3, and sst_4 (Khare et al. 1999). This suggests that somatostatin action on rat vascular injury is mediated through one of these receptors. This is also in agreement with the results obtained in this Thesis (II, III). The sst_1/sst_4 subtype-selective analog CH275 and the sst_4-selective L-803,087 significantly inhibited neointimal hyperplasia after vascular injury. Similar to octreotide and lanreotide in clinical studies, the subtype-selective analogs for sst_2, sst_3, and sst_5 failed to shown any vasculoprotective characters and the sst_1-selective analog had only a moderate effect on neointimal hyperplasia. The results from the *ex vivo* tissue cultures support these findings, and together all suggest targeting through sst_4 when aiming at vasculoprotection with somatostatin analogs (II, III).

Even if the homology between rat and human somatostatin receptors is high, ranging from 82% (sst_5) to 94% (sst_1) (Patel et al. 1995), there are significant species dependent differences in binding affinities between different somatostatin receptors for somatostatin analogs. As an example, the rat sst_5 has a 160-fold higher affinity for octreotide than the human sst_5 (O'Carroll et al. 1994). The affinities of the non-peptidyl sst subtype-specific ligands used in this study (III) were originally tested with human receptors (Rohrer et al. 1998), but they have successfully been used also in rat studies (Parmar et al. 1999, Pittaluga et al. 2000, Blake 2001, Cowles et al. 2002, Elberg et al. 2002, Emery et al. 2002, Stark and Mentlein 2002, Cervia et al. 2003). Also the sst_1/sst_4 ligand used, CH275, has been widely and successfully used in rat models (Chen et al. 1997b, Traina et al. 1998, Mastrodimou et al. 2004, Marazioti et al. 2005). Still, results obtained with rat models can not be transferred to man directly, and will require verification in clinical trials before any further conclusions can be made.

The vasculoprotective effects of somatostatin and its analogs

Several mechanisms have been suggested to account for the vasculoprotective effect of somatostatin. It has been shown that sst_2/sst_3/sst_5 analogs inhibit SMC proliferation and migration through inhibition of growth factors, such as PDGF, IGF-1 and bFGF (Häyry et al. 1993b, Mennander et al. 1993, Grant et al. 1994, Lauder et al. 1997), or by affecting autocrine and paracrine mechanisms that regulate cell replication (Lundergan et al. 1991). Also, a direct sst_2-mediated inhibition of vascular SMC proliferation has been suggested (Grant et al. 1994, Grant and Caballero 2002). Furthermore, sst_2/sst_3/sst_5 analogs have been shown to inhibit neointimal hyperplasia through inhibition of growth hormone release (Tiell et al. 1978, Bruns et al. 2000).

Very little is known about vascular effects mediated through sst_4. Somatostatin has been suggested to exert an anti-inflammatory effect on the vascular endothelium through sst_4 (Badway et al. 2004). In our model of vascular injury the importance of this mechanism was most likely limited, as the endothelium was completely removed by a balloon catheter, and the first neointimal cells appeared at day 4 post injury, while the explants for the *ex vivo* cultures were obtained already at day 2 post injury (II, III). Also, in the *in vivo* experiments, drug treatment was stopped on day 14 post injury (II, III), when the reendothelialization was not completed (I).

The role of sst_4 in mediating an anti-migratory effect on neointimal cells has previously been implicated based on the increase in sst_4 expression post injury at the time when the migratory activity in the vessel wall is strongest (Khare et al. 1999). In agreement with this finding, both the sst_1/sst_4-selective analog CH275, and the sst_4-selective ligand L-803,087 showed anti-migratory properties in an *ex vivo* setting with vessel cultures (II, III). It can be speculated that as the *ex vivo* anti-migratory effect was seen early during the time-point of progenitor cell influx (I, II, III), targeting through sst_4 would inhibit the migration of these cells to the vascular wall.

In the *in vitro* thymidine incorporation assay, the sst_1 analog showed antiproliferative properties on a primary rat SMC cell line, and the A10 cell line from rat embryonic thoracic aorta. An anti-proliferative effect was also seen to a lesser extent on a human skin fibroblast and the MCF-7 human mammary adenocarcinoma cell lines, both of which have been reported to express all five ssts (Hagstromer et al. 2006, Watt and Kumar 2006). The results were only seen with quite high doses of the analog, 5 and 10 μM, but toxicity was not observed, as withdrawal of the drug resulted in a catch-up in proliferation (Tigerstedt NM et al. unpublished results). Nevertheless, the high doses of the sst_1 analog needed for the inhibition of proliferation might refer to an off-target response. In the *ex vivo* explant assay, none of the tested compounds inhibited cell proliferation (II, III).

Although there is still much to learn about the biology of somatostatin and somatostatin receptors, the results in this study (II, III) are in agreement with the current literature. The predominant sst receptors in the rat model of vascular injury used in this thesis are sst_1, sst_3 and sst_4 (Khare et al. 1999). The first somatostatin study showed the superiority of an sst_1/sst_4-selective agonist in inhibiting intimal hyperplasia both *in vivo* and *ex vivo*, and narrowed down the interest to two sst subtypes: sst_1 and sst_4 (II). When targeted separately, only sst_4 showed vasculoprotective properties, and this effect seems to

be mediated through inhibition of migration rather than proliferation (II, III). These results offer an explanation for why $sst_2/sst_3/sst_5$ analogs failed to prevent restenosis in clinical trials. Already a brief 14-day treatment post injury was sufficient for vasculoprotection (II, III), providing evidence for the importance of early therapeutic intervention when aiming to prevent intimal hyperplasia.

As the classical somatostatin effects are mediated through $sst_2/sst_3/sst_5$, the most common adverse effects with analogs targeting these receptors are gallstones, diarrhea, nausea, abdominal pain, and hypoglycemia or hyperglycemia. During long-term octreotide treatment gallstones can occur in as many as 50% of the patients treated (Dowling et al. 1992). It would thus be important to be able to bypass these adverse effects by selective receptor targeting. However, if the vasculoprotective properties of somatostatin are mediated through sst_4 (II, III), we might speculate that by targeting through sst_4 only, adverse effects associated with $sst_2/sst_3/sst_5$ analogs could be avoided.

3.2 Sirolimus and imatinib

Sirolimus-eluting stents significantly reduce restenosis rates after PTCA (Morice et al. 2002, Moses et al. 2003, Morice et al. 2007). However, the currently considered safe use of SESs is limited to very specific vessel and lesion types and requires prolonged anti-thrombotic medication (Laskey et al. 2007), and it is thus not a treatment suitable for all patients or all vascular fibroproliferative disorders. Oral sirolimus treatment has been limited by side effects related to the high drug doses needed for restenosis inhibition (Waksman et al. 2004).

As imatinib inhibits SMC proliferation and vascular progenitor cell action, but not the proliferation of endothelial cells (Gambacorti-Passerini et al. 1997, Wang et al. 2006, Hacker et al. 2007), it possesses many qualities of a theoretical "ideal" antirestenosis drug. In rodent models, imatinib has shown some vasculoprotective potential, most likely through its inhibitory effects on the PDGFR and c-kit tyrosine kinases (Myllärniemi et al. 1999, Sihvola et al. 2003, Lassila et al. 2004, Wang et al. 2006). These affect SMC accumulation, migration, proliferation, dedifferentiation, as well as stem cell proliferation, adhesion, and mobilization (Ferns et al. 1991, Jawien et al. 1992, Leary et al. 1992, Fleming et al. 1993, Levesque et al. 1995, Kenagy et al. 1997).

The clinical experience on imatinib and its vasculoprotective properties is limited to a pilot study, where imatinib failed to prevent recurrent in-stent restenosis in a group of 180 patients at 1 year after stent implantation (Zohlnhofer et al. 2005). In that study, imatinib treatment was initiated 2 days before repeat intervention, and continued for 10 days. This lack of long-term vasculoprotection by short-term interference with PDGF signaling has been implied in studies with other PDGF inhibitors as well (Leppänen et al. 2000). These findings are in line with the results in this thesis study: short-term imatinib treatment did not produce long-term inhibition of intimal hyperplasia, and a catch-up in intimal growth was seen already 3 weeks after discontinuation of the treatment (IV). Thus, imatinib monotherapy might not be powerful enough for the prevention of neointimal hyperplasia, at least with the brief early exposure as the treatment strategy.

The synergistic effect of sirolimus and imatinib

Optimal long-term vasculoprotection might require simultaneous targeting of several signaling pathways involved in neointimal development. Several drug combinations have been suggested for dual therapies. In the clinic, sirolimus has been successfully combined both with CsA (Kahan et al. 1998), and tacrolimus (McAlister et al. 2000) to improve the outcome of allograft rejection, and with fluvastatin in rodent models (Gregory et al. 2001). In preclinical cancer treatment studies, imatinib has been combined with sirolimus to overcome drug resistance due to new mutations in Bcr-Abl (Ly et al., 2003, Mohi et al. 2004).

In a rat denudation injury model, inhibiting cell cycle progression with sirolimus and the c-kit and PDGF-R kinases with imatinib for the first 14 days after denudation injury resulted in a synergistic suppression of intimal hyperplasia (Vamvakopoulos et al. 2006). In this thesis study it was further shown that this well-timed and well-tolerated oral combination treatment with submaximal doses of sirolimus and imatinib produced a long-term synergistic suppression of intimal hyperplasia, reducing intimal area by 80% even at 90 days after injury (IV).

The mechanisms behind the synergistic effect have not been established earlier. With gene expression analyses, we were able to show that the combination treatment upregulated the mRNA expression of SMC markers CSRP2 and SM22α (IV). This most likely reflects an inhibition of SMC dedifferentiation, or a promotive effect on SMC integrity, as both CSRP2 and SM22α are associated with the maintenance of SMCs in a differentiated, contractile, and passive state (Louis et al. 1997, Zhang et al. 2001, Owens et al. 2004, Wei et al. 2005). The phenotypic switch of SMCs to dedifferentiated secretory SMCs is a hallmark feature of intimal hyperplasia (Shanahan et al. 1993, Shanahan et al. 1994), and in vehicle-treated rats, the expression of both CSRP2 and SM22α mRNA was sharply downregulated immediately after denudation injury (IV). Furthermore, stabilizing cell integrity and inhibiting SMC apoptosis have recently been proposed as a strategy to prevent intimal hyperplasia (Sukhanov et al. 2007). As the combination treatment also upregulated the anti-apoptotic BCL2 gene, this finding might be of significance for the vasculoprotective effects of this treatment.

Several growth factors are involved in the control of the SMC phenotypic switch, including transforming growth factor-ß (TGF-ß) (Muto et al. 2007), and PDGF-BB (Dandre and Owens 2004, Liu et al. 2005). As sirolimus increases TGF-ß expression (Wang et al. 1996), and imatinib inhibits PDGF receptor kinases (Buchdunger et al. 2000), the effects of the combination treatment on SMCs might be related to growth factor mediated signaling events.

Compared to the other treatment groups the combination treatment was also more efficient in inhibiting neointimal cell migration in the *ex vivo* explant model, while sirolimus showed the highest antiproliferative capacity (IV). As the contribution of cell migration may be more important in the development of intimal hyperplasia than that of cell proliferation (I; Du Toit et al. 2001), the anti-migratory properties of the combination treatment might explain its superior *in vivo* effects.

Sirolimus is a powerful antiproliferative agent. However, as it inhibits neointimal hyperplasia, it also interferes with the vascular healing process after stenting by inhibiting endothelial cell proliferation and slowing down reendothelialization, thus increasing the risk for stent thrombosis (Virmani et al. 2004, Iakovou et al. 2005). In the study with the combination of sirolimus and imatinib (IV), one concern was how this combination would affect reendothelialization post injury. As expected, a downregulation of the endothelial marker von Willebrandt factor mRNA expression was seen at 40 days post injury in the rats receiving sirolimus treatment, and the number of vWF-positive luminal cells was reduced. In the combination treatment group, an even stronger downregulation of vWF mRNA expression was observed. PDGF-R kinase-directed compounds, such as imatinib, should not affect endothelial cells (Levitzki 2005). On the contrary, inhibition of PDGF-R-ß has been shown to promote reendothelialization (Noiseux et al. 2000), and it could thus have been anticipated that the addition of imatinib to the sirolimus treatment would enhance reendothelialization. However, as sirolimus inhibits endothelial progenitor cell proliferation (Fukuda et al. 2005), and imatinib also targets the SCF/c-kit pathway (Wang et al. 2006), the combination therapy might have an additive effect on recruitment, proliferation, or migration of endothelial progenitor cells.

In concert with previous findings (Aavik et al. 2005), the most intense gene expression changes were seen during the first weeks post-injury (IV). Furthermore, the influx of neointimal progenitor cells occurs already at 2-4 days post injury (I). Thus, as shown with both the combination of sirolimus and imatinib (IV), as well as with sst analogs targeting sst_4 (II, III), an early timed therapeutical intervention is crucial when aiming at vasculoprotection.

CONCLUSIONS

The following conclusions can be drawn from this study:

1. Whole vessel vascular explant cultures can be used to study the migration and proliferation of neointimal cells in response to *in vivo* denudation injury, without excluding the participation of neointimal progenitor cells. This assay model can also be used to evaluate the anti-migratory and anti-proliferative properties of potential vasculoprotective compounds.

2. In rat, the vasculoprotective effects of somatostatin are mediated through sst_4, and this effect is mediated through inhibition of cell migration rather than proliferation. Thus, clinical trials with sst_2/sst_3/sst_5-selective analogs failed because the wrong receptor subtypes might have been targeted. Targeting through sst_4 should be considered when developing somatostatin-based vasculoprotective therapies.

3. The combination of sirolimus and imatinib at well-tolerated submaximal doses provides a long-term synergistic inhibition of neointimal hyperplasia in rat. The mechanisms behind the synergy most likely include inhibition of post-operative thrombocytosis and leukocytosis, inhibition of neointimal cell migration to the injury-site, and maintenance of cell integrity by inhibition of apoptosis and SMC dedifferentiation.

4. The outer vascular layer, adventitia, is more than just a passive supportive structure, and it contributes actively in vascular repair. The influx of circulating progenitor cells occurs already during the first days post-injury, and the migration of neointimal cells to the site of injury is a hallmark feature of neointimal hyperplasia. Thus, inhibition of cell migration is more important than interfering with cell proliferation.

5. Together these findings underline the importance of early therapeutic intervention with anti-migratory compounds in preventing intimal hyperplasia. The vasculoprotective potential of sst_4 analogs and the combination of sirolimus and imatinib could serve as a basis for future therapeutic considerations.

YHTEENVETO (FINNISH SUMMARY)

Verisuoniahtauman pallolaajennuksen jälkeen jopa 30-50% laajennetuista suonista ahtautuu uudelleen 3-6 kuukauden sisällä suonten sisäkerroksen, intiman, paksuuntumisen seurauksena. Valtimoiden uudelleen ahtautumisen, eli restenoosin kehittymisessä avainasemassa ovat erilaistumattomat sileälihassolut, jotka aktivoituvat verisuonivaurion seurauksena ja kerääntyvät hallitsemattomasti verisuonen sisäpinnalle. Aikaisemmin sileälihassolujen uskottiin vaeltavan intimaan verisuonen keskikerroksesta, mediasta. Viime aikoina julkaistu tutkimustieto kuitenkin osoittaa näiden solujen olevan ainakin osittain peräisin verenkierron kantasoluista, mikä on luonut uusia haasteita valtimoiden uudelleen ahtautumisen tutkimukselle, koska sileälihassolujen käyttäytymistä on perinteisesti tutkittu valtimon keskikerroksesta peräisin olevien sileälihassoluviljelmien avulla.

Somatostatiini on viiden G-proteiinikytketyn reseptorin (sst_1-sst_5) kautta vaikuttava solukasvua rajoittava hormoni. Ihmisen verisuonissa on lähes yksinomaan reseptorialatyyppejä 1 ja 4 ja rotalla näiden reseptoreiden määrän on todettu lisääntyvän äkillisesti verisuonivaurion jälkeen. Eläinmalleissa somatostatiini estää pallolaajennuksen jälkeistä restenoosia. Kliinisissä kokeissa sst_2/sst_3/sst_5 -selektiiviset analogit oktreotidi ja lanreotidi eivät kuitenkaan pystyneet merkittävästi estämään sepelvaltimoiden restenoosia.

Sirolimuusi on mTOR-estäjä, jonka on osoitettu sekä eläinmalleissa että kliinisissä kokeissa estävän pallolaajennuksen jälkeistä verisuonten uudelleen ahtautumista. Systeemisen sirolimuusihoidon ongelmana on kuitenkin ollut lääkkeen huono siedettävyys annoksilla, joita vaaditaan hoitotehon saavuttamiseksi. Imatinibi on tiettyjen syöpien hoidossa käytetty tyrosiinikinaasi-estäjä, jonka on eläinkokeissa osoitettu omaavan myös suotuisia verisuonivaikutuksia. Rotalla imatinibin ja sirolimuusin yhdistelmähoito estää synergistisesti pallolaajennuksen jälkeistä verisuonten uudelleenahtaumaa jo hyvin siedetyillä matalilla annoksilla. Synergian taustoja tai pitkäaikaistehoa ei kuitenkaan tunneta.

Tämän väitöskirjatyön tavoitteena oli kehittää *ex vivo* – kudosviljelymenetelmä, jonka avulla pystyttäisiin tutkimaan valtimon uudelleenahtaumaan osallistuvien solujen toimintaa sulkematta pois kantasolujen vaikutusta prosessissa. Tavoitteena oli lisäksi selvittää somatostatiinianalogien sekä sirolimuusi-imatinibi -yhdistelmähoidon verisuonivaikutuksia. *Ex vivo* kudosviljelmien lisäksi työssä käytettiin soluviljelmiä, rotan kaulavaltimon ja aortan pallolaajennusmalleja, immunohistokemiaa sekä kvantitatiivista real-time PCR-menetelmää.

Tämä tutkimustyö osoittaa, miten *ex vivo* –kudosviljelymenetelmää voidaan käyttää verisuonen uudelleenahtaumaan osallistuvien solujen tutkimiseen (I), sekä lääkeaineiden verisuonivaikutusten arviointiin (I-IV). Työssä havaittiin, että erilaistumattomat kantasolut hakeutuivat verisuoneen heti ensimmäisinä verisuonivaurion jälkeisinä päivinä, ja että solujen vaeltaminen (migraatio) verisuonen seinämässä oli verisuonivaurion korjausprosessin kannalta solujen jakautumista (proliferaatio) tärkeämpää (I). Lisäksi adventitian todettiin osallistuvan aktiivisesti verisuonivaurion korjaamiseen (I).

Somatostatiinianalogeista sst_1/sst_4 -selektiivinen CH275 esti rotan verisuonten uudelleenahtaumaa merkittävästi sst_2/sst_3/sst_5 -selektiivisiä analogeja tehokkaammin (II).

Reseptoriselektivisten lääkeaineiden avulla osoitettiin somatostatiinin verisuonia suojaavan vaikutuksen välittyvän erityisesti sst_4:n kautta (III). Tämä tapahtui pääosin estämällä solujen vaeltamista, ei niinkään solujen jakautumista. Väitöskirjassa osoitetaan lisäksi, että oikein ajoitettu sirolimuusin ja imatinibin yhdistelmähoito säilytti tehonsa myös pitkäaikaisseurannassa. Yhdistelmähoidon vaikutus perustui leikkauksen jälkeisen trombosytoosin, leukosytoosin sekä solujen vaeltamisen estoon, sileälihassolujen erilaistumisasteen ylläpitämiseen sekä apoptoosin estoon (IV).

Väitöskirjatyö osoittaa restenoosiin osallistuvien solujen ilmaantuvan verisuonen seinämään heti ensimmäisinä verisuonivaurion jälkeisinä päivinä ja solujen vaeltamisen olevan tässä prosessissa solujen jakautumista tärkeämpää. Tämän vuoksi verisuonten uudelleen ahtaumista on yritettävä hoitaa solujen vaeltamista estävillä lääkeaineilla välittömästi verisuonivaurion jälkeen. Sekä sst_4–selektiiviset lääkeaineet että sirolimuusin ja imatinibin yhdistelmä ovat varteenotettavia vaihtoehtoja tämänkaltaisten verisuoniselektiivisten lääkeaineiden kehittelyssä.

SAMMANDRAG (SWEDISH SUMMARY)

Efter ballongdilatation av aterosklerotiska artärer återförträngs 30-50% av blodkärlen inom 3-6 månader på grund av förtjockning av artärväggens innersta lager, intiman. En central roll i återförtjockningen av artärer, eller restenosen, utspelas av dedifferentierade glatta muskelceller, som okontrollerat ansamlas på insidan av den skadade artärväggen. Tidigare uppgavs glatta muskelceller migrera till intiman från artärens mellanlager, median. Nyligen har det dock påvisats, att en betydande del av de glatta muskelceller som ansamlas vid artärskadan kan härstamma från cirkulerande stamceller. Det här har medfört nya utmaningar för forskningen av restenos, då man traditionellt undersökt glatta muskelcellers beteende med hjälp av cellodlingar som härstammat från artärens mellanlager.

Somatostatin är ett neurohormon som agerar via fem G-proteinkopplade receptorer (sst_1-sst_5) och hämmar celltillväxt. I människans blodkärl förekommer nästan enbart sst_1 och sst_4, och hos råttor har mängden av de här receptortyperna konstaterats öka akut efter en blodkärlsskada. I djurmodeller har somatostatin och dess analoger kunnat hämma artärers återförtjockning efter ballongdilatation. I kliniska försök med de sst_2, sst_3 och sst_5-selektiva somatostatinanalogerna oktreotid och lanreotid kunde dock just ingen blodkärlsskyddande effekt påvisas.

Sirolimus är en mTOR-hämmare som minskar återförtjockning av artärer efter ballongdilatation både i försöksdjursmodeller och i kliniska studier. Hos människor har dock sirolimusvården associerats med med märkbara biverkningar med de höga doserna som krävts för att den önskade vårdeffekten kunnat uppnås. Imatinib är en tyrosinkinashämmare som används vid vård av vissa cancertyper. Hos råttor har imatinib haft blodkärlsskyddande effekter. Därtill har man kunnat påvisa, att kombinationen av imatinib och sirolimus hämmar återförträngning av artärer synergistiskt redan med vältolererade submaximala doser av läkemedlen. Kombinationsmedicineringens långtida effekt och mekanismerna bakom synergin är dock okända.

Målet med den här avhandlingen var att utvecka en *ex vivo*–vävnadsodlingsmetod, med vilken man kunde undersöka funktionen av celler som deltar i restenos utan att utesluta inverkan av stamceller. Målet var därtill att utforska de blodkärlsskyddande effekterna bakom somatostatinanaloger och kombinationsbehandlingen med sirolimus och imatinib. Förutom *ex vivo*-vävnadsodlingar användes cellodlingar, ballongdilatations-modeller med råttor, immunohistokemi samt kvantitativ real-time PCR.

Den här avhandlingsstudien påvisar hur en *ex vivo*–vävnadsodlingsmetod kan användas för att studera de celler som deltar i artärers återförtjockning (I). Modellen kan även användas för att utvärdera potentiella läkemedelsbehandlingars antimigratoriska och antiproliferativa effekter (I-IV). Därtill påvisas, att stamceller söker sig till artärväggen redan under de första dagarna efter blodkärlsskadan, att cellmigration spelar en viktigare roll än cellproliferation under artärväggens läkningsprocess och att adventitian aktivt deltar i restenos (I).

Den sst_1/sst_4-selektiva somatostatinanalogen CH275 kan hämma artärers återförtjockning efter ballongdilatation betydligt bättre än sst_2/sst_3/sst_5-selektiva somatostatinanaloger (II). Med hjälp av analoger selektiva för var sin sst specificeras

därtill, att somatostatinets blodkärlsverkan medieras i första hand via sst_4. Den här effekten sker främst via inhibition av cellmigration, inte cellproliferation (III). Dessutom påvisar avhandlingsarbetet, att effekten av en tidig och kortvarig kombinationsbehandling med vältolererade submaximala doser av sirolimus och imatinib är långvarig. Kombinationsbehandlingens effekt grundar sig på hämmandet av post-operativ trombocytos och leukocytos, samt hämmandet av cellmigration och glatta muskelcellers dedifferentiation och apoptos (IV).

Avhandlingen påvisar, att neointimans stamceller strömmar in i blodkärlsväggen redan under de första dagarna efter blodkärlsskadan och att cellmigration spelar en större roll än cellproliferation i återförtjockningsprocessen. Därför bör försök att hindra artärers återförtjockning påbörjas omedelbart efter blodkärlsskadan med antimigratoriska läkemedel. Somatostatinanaloger selektiva för sst_4 och kombinationen med sirolimus och imatinib kan representera sådana behandlingsalternativ.

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